

Annexins: From Structure to Function

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Gerke, Volker, and Stephen E. Moss. Annexins: From Structure to Function. *Physiol Rev* 82: 331–371, 2002; 10.1152/physrev.00030.2001.—Annexins are Ca^{2+} and phospholipid binding proteins forming an evolutionary conserved multigene family with members of the family being expressed throughout animal and plant kingdoms. Structurally, annexins are characterized by a highly α -helical and tightly packed protein core domain considered to represent a Ca^{2+} -regulated membrane binding module. Many of the annexin cores have been crystallized, and their molecular structures reveal interesting features that include the architecture of the annexin-type Ca^{2+} binding sites and a central hydrophilic pore proposed to function as a Ca^{2+} channel. In addition to the conserved core, all annexins contain a second principal domain. This domain, which NH_2 -terminally precedes the core, is unique for a given member of the family and most likely specifies individual annexin properties *in vivo*. Cellular and animal knock-out models as well as dominant-negative mutants have recently been established for a number of annexins, and the effects of such manipulations are strikingly different for different members of the family. At least for some annexins, it appears that they participate in the regulation of membrane organization and membrane traffic and the regulation of ion (Ca^{2+}) currents across membranes or Ca^{2+} concentrations within cells. Although annexins lack signal sequences for secretion, some members of the family have also been identified extracellularly where they can act as receptors for serum proteases on the endothelium as well as inhibitors of neutrophil migration and blood coagulation. Finally, deregulations in annexin expression and activity have been correlated with human diseases, e.g., in acute promyelocytic leukemia and the antiphospholipid antibody syndrome, and the term *annexinopathies* has been coined.

I. INTRODUCTION: OVERVIEW OF THE ANNEXIN FAMILY

Nature has achieved the means to tightly control intracellular Ca^{2+} concentrations, thereby enabling the

ion to serve second messenger functions in a variety of processes which couple extracellular signals to cellular responses. Systems regulating intracellular Ca^{2+} levels thus are considered part of the intricate Ca^{2+} signaling network. They include gated Ca^{2+} channels and energy-

dependent pumps, which are located in organelle membranes and the plasma membrane, as well as intracellular Ca^{2+} binding proteins serving as regulated Ca^{2+} buffers. Other classes of Ca^{2+} binding proteins participate more directly in Ca^{2+} signaling as they display altered properties in response to Ca^{2+} binding. Annexins can be considered a subgroup of the latter, although their precise position within Ca^{2+} signaling chains remains elusive. Moreover, a growing body of evidence suggests that annexins can also function in their Ca^{2+} -free conformation in a hitherto unknown fashion, thereby increasing the functional diversity among these proteins.

The name annexin is derived from the Greek *annex* meaning "bring/hold together" and was chosen to describe the principal property of all or at least nearly all annexins, i.e., the binding to and possibly holding together of certain biological structures, in particular membranes. The name also has a somewhat historical flavor as it takes into account the point that a number of the groups who independently of one another discovered annexins were in search for such scaffolding or bridging proteins. However, initially, i.e., at the date of their discoveries in the late 1970s and early 1980s, annexins received diverse and unrelated names referring to their biochemical properties. These included synexin (for granule aggregating protein, Ref. 52), chromobindins (proteins binding to chromaffin granules, Ref. 54), calcimedins (proteins mediating Ca^{2+} signals, Ref. 199), lipocortins (steroid-inducible lipase inhibitors, Ref. 85), and calpactins (proteins binding Ca^{2+} , phospholipid, and actin, Ref. 101). Intensive biochemical work, protein and cDNA sequencing, as well as gene cloning led to the realization that all such proteins identified shared key biochemical properties as well as gene structure and sequence features. Hence, the concept of a novel multigene family arisen by gene duplication was developed and the common name annexin was introduced to solve the terminology tangle (55).

By definition, an annexin protein has to fulfill two major criteria. First, it must be capable of binding in a Ca^{2+} -dependent manner to negatively charged phospholipids. Second, it has to contain as a conserved structural element the so-called annexin repeat, a segment of some 70 amino acid residues. Molecular structures obtained for a number of annexins over the past decade helped to extend the similarities to the three-dimensional level. Moreover, they defined a hitherto unknown structural fold, the conserved annexin domain, which is built of four annexin repeats packed into a highly α -helical disk, and which now is considered to be a general membrane binding module. Once clearly defined and advanced by genome sequencing work, the annexin family has grown steadily in the 1990s, and with the turn of the century, now amounts to more than 160 unique annexin proteins present in more than 65 different species ranging from fungi and protists to plants and higher vertebrates (Fig. 1)

(202, 204). In this review we summarize the biochemical and structural properties of annexins, putting a particular emphasis on novel aspects of annexin interactions with lipids and other biological ligands. For a detailed discussion of the canonical annexin properties, their structural organization, and intracellular as well as tissue distribution, the interested reader is referred to previous reviews (51, 97, 244).

Having accumulated a wealth of biochemical and structural knowledge, we are still in need of assigning a physiological function to the annexin family as a whole, or better, because they are likely to differ, to individual annexins. Recent knock-out models, both at the cellular and the animal level, as well as the development and use of dominant-negative mutant proteins have introduced the first direct approaches for analyzing annexin function. They underscore the concept of functional diversity within the family. Moreover, it has recently become clear that certain dysregulations in annexin expression and activity can be correlated with human diseases and that this has led to the introduction of the term *annexinopathies*. Although we still have to await final proof of a direct correlation, we decided to concentrate our review on such recent developments leading to the proposal of some models concerning annexin function.

II. BIOCHEMICAL PROPERTIES OF ANNEXINS AND THEIR THREE-DIMENSIONAL STRUCTURE

A. Molecular Structures

1. *Structures of annexin protein cores: the conserved membrane binding modules*

Each annexin is composed of two principal domains: the divergent NH_2 -terminal "head" and the conserved COOH -terminal protein core. The latter harbors the Ca^{2+} and membrane binding sites and is responsible for mediating the canonical membrane binding properties. An annexin core comprises four (in annexin A6 eight) segments of internal and interannexin homology that are easily identified in a linear sequence alignment (for review, see Ref. 244). It forms a highly α -helical and tightly packed disk with a slight curvature and two principle sides. The more convex side contains novel types of Ca^{2+} binding sites, the so-called type II and type III sites (335), and faces the membrane when an annexin is associated peripherally with phospholipids. The more concave side points away from the membrane and thus appears accessible for interactions with the NH_2 -terminal domain and/or possibly cytoplasmic binding partners (Fig. 2). The first structure known for an annexin core was that solved by Huber et al. (134) for annexin A5 in 1990. In the

Name	Synonyms/Former name(s)	Human gene symbol	Non-human gene symbol
annexin A1	lipocortin 1, annexin I	ANXA1	Anxa1
annexin A2	calpactin 1, annexin II	ANXA2	Anxa2
annexin A3	annexin III	ANXA3	Anxa3
annexin A4	annexin IV	ANXA4	Anxa4
annexin A5	annexin V	ANXA5	Anxa5
annexin A6	annexin VI	ANXA6	Anxa6
annexin A7	synexin, annexin VII	ANXA7	Anxa7
annexin A8	annexin VIII	ANXA8	Anxa8
annexin A9	annexin XXXI	ANXA9	Anxa9
annexin A10		ANXA10	Anxa10
annexin A11	annexin XI	ANXA11	Anxa11
annexin A12	unassigned		
annexin A13	annexin XIII	ANXA13	Anxa13
HUMAN ANNEXINS plus COGNATE ORTHOLOGS			
? Aplysia			
Name	Organism/Former name	Gene symbol	
annexin B9	3 species of insect, annexin IX	Anxb9	
annexin B10	4 species of insect, annexin X	Anxb10	
annexin B11	1 species of insect, annexin	Anxb11	
annexin B12	Cnidaria, annexin XII	Anxb12	
ANIMAL ANNEXINS without HUMAN ORTHOLOGS			
Name	Organism/Former name	Gene symbol	
annexin C1	Dictyostelium and Neurospora annexin XIV	Anxc1	
annexin C2-C5	4 species of fungi/ molds/algaeolates	Anxc2-c5	
FUNGI/MOLDS and CLOSE RELATIVES			
Name	Organism/Former name	Gene symbol	
annexin D1-D25	35 species including annexin XVIII and annexins XXII-XXIX	Anxd1-d25	
PLANTS			
Name	Organism/Former name	Gene symbol	
annexin E1	Giardia annexin XXI	Anxe1	
annexin E2	Giardia annexin XIX	Anxe2	
annexin E3	Giardia annexin XX	Anxe3	
PROTISTS			

FIG. 1. The new annexin nomenclature. The five major annexin groups (A–E) are shown, with details of the most extensively studied family members. The nomenclature is that proposed by Reg Morgan and Pilar Fernandez and endorsed by participants at the 50th Harden Conference on Annexins held at Wye College, UK, September 1–5, 1999. A more extensive list of annexin subfamilies and species is posted at the European annexin web site (<http://www24.brinkster.com/annexins/>). There are several important points to note. The vertebrate annexins (A1–A13) are unlikely to be widely represented in invertebrate species. The oldest of this group, namely, annexins A7, A11, and A13, are possible exceptions, and an annexin A11 ortholog has been described in the mollusk *Aplysia*. Within the B group, the *Caenorhabditis elegans* annexins have yet to be assigned numbers. In the C group, the *Dictyostelium* annexin, originally described incorrectly as annexin VII (synexin), is now established as being orthologous to the *Neurospora* annexin.

meantime, more than 10 crystal structures for annexin cores have been described showing a remarkable conservation of the overall three-dimensional fold. Aspects of molecular annexin structures have been reviewed in detail previously (see, for example, Refs. 133, 178, 305), and

the purpose of this review is to discuss only recent and novel developments in this area.

Recent findings include the elucidation of the first structures of annexins from lower eukaryotes and plants. Liemann et al. (177) crystallized the core of annexin C1

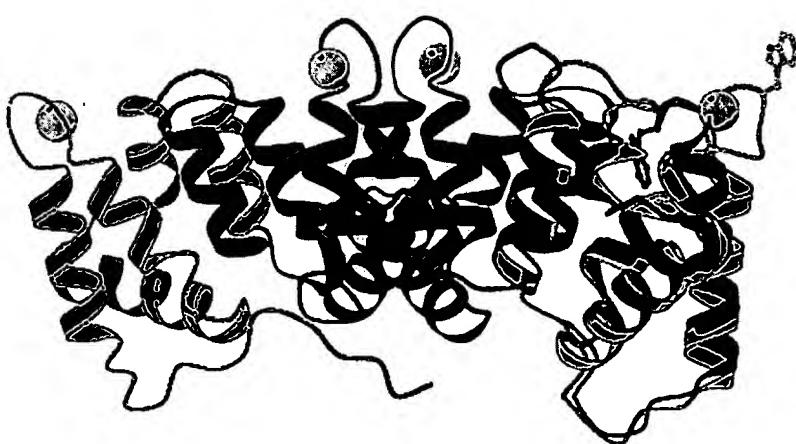


FIG. 2. Crystal structure of human annexin A5. The ribbon drawing illustrates the highly α -helical folding of the protein core that forms a slightly curved disk. Different colors were chosen to highlight the four annexin repeats that are given in green (repeat I), blue (repeat II), red (repeat III), and violet/cyan (repeat IV). The NH_2 -terminal domain appears unstructured and extends along the concave side of the molecule (green). The high and low Ca^{2+} forms are shown in a superposition revealing the conformational change in repeat III, which leads to an exposure of Trp-187 (violet for the low and cyan for the high Ca^{2+} form). Bound Ca^{2+} are depicted as yellow spheres. [Image kindly provided by R. Huber, S. Liemann, and A. Lewitt-Bentley, as modified from Ref. 178.]

from *Dictyostelium discoideum*, whereas Hofmann et al. (127) elucidated the structure of a plant annexin, annexin D11 from *Capsicum annuum*, which revealed not only the typical annexin fold but also differences to nonplant annexins in annexin repeats I and III and in the membrane binding loops. Another recent advance is the introduction of benzodiazepine and benzothiazepine derivatives as annexin ligands and their cocrystallization with annexins. The benzothiazepine K201 was first described to bind to annexin A5 and inhibit its Ca^{2+} channel activity, most likely by restraining a hinge movement of the two annexin A5 modules formed by annexin repeats I/IV and II/III, respectively (149, 150). Other structurally related benzodiazepine compounds have subsequently been identified as ligands for various annexins with the interaction being based on similar structural principles (126). However, a possible pharmacological role of these interactions remains to be shown. Crystal structure determination and biochemical characterization in combination with site-directed mutagenesis have also proven powerful in recent years in characterizing the contribution of certain residues to the overall fold of annexin cores and/or their biochemical properties. Conserved arginine residues present in the so-called endonexin fold of each homology segment, for example, were shown to be crucial for stabilizing the tertiary structure of annexin A5. On the other hand, substitution by alanine of different serine and threonine residues and the unique tryptophan in the same annexin results in altered membrane binding underscoring the importance of these residues in mediating intermolecular, i.e., annexin-phospholipid, contacts (31, 32). Moreover, mutational analysis revealed that the aspartate residue at position 226 of annexin A5 participates as a molecular switch in a Ca^{2+} - and pH-dependent conformational change (294). Like many other annexins, annexin A4 is a substrate of protein kinase C (PKC), at least in *in vitro* reactions, and Kaetzel et al. (147) have attempted to monitor structural changes resulting from this phosphorylation. They show that replacement by glutamate of the PKC acceptor site, threonine-6, causes a release of the NH_2 -terminal domain from the protein core indicative of a regulatory role in the membrane aggregation displayed by this annexin. Annexin B12 has also been subjected to detailed scrutiny by mutagenesis involving cysteine substitutions for spin labeling purposes (see below) and the glutamate at position 105. This residue has been found to participate in the formation of intermolecular Ca^{2+} binding sites in a hexameric form of the molecule (185), and replacement of Glu-105 by lysine stabilizes this hexamer by favoring extensive hydrogen bonding (35).

Recently, techniques other than crystallization of the soluble proteins have been introduced to study in detail structural properties of annexins, in particular when

bound to membrane or phospholipid surfaces. They include cryoelectron microscopy, which led to the identification of highly structured junctions formed by different annexins between opposing membranes (167), and atomic force microscopy (AFM), which enabled the high-resolution analysis of two-dimensional crystals of annexin A5 formed on planar lipid bilayers (248, 249). Two-dimensional crystals of annexin A6 formed on artificial lipid monolayers were also obtained and characterized recently, revealing an intrinsic flexibility of this eight-annexin repeat-containing molecule. Here the two lobes of annexin A6, i.e., repeats I-IV and V-VIII, respectively, were found to bind to the phospholipid in both parallel or antiparallel orientation, with the latter providing a structural basis for membrane cross-linking (6). Evidence for conformational changes occurring in annexins upon membrane binding was obtained by analyzing membrane-bound annexin A5 with transmission and internal reflection infrared spectroscopy. Interestingly, it was inferred from these studies that a new β -structure with interstrand hydrogen bonds oriented parallel to the membrane surface is formed upon interaction with a lipid monolayer. On the other hand, analyses of two-dimensional crystals of the same annexin on membrane surfaces by high-resolution electron microscopy and AFM, and crystal structure analysis of a cross-linked form of annexin A2 capable of binding membranes, do not provide evidence for substantial conformational alterations accompanying the canonical Ca^{2+} -dependent membrane binding (26, 29, 229, 248). Relatively subtle changes, however, might occur. These include the exposure of the unique tryptophan in repeat 3 of annexin A5 observed in high Ca^{2+} (47, 174). Thus, despite the wealth of structural information on soluble as well as membrane-bound annexins, it is not clear whether the peripheral and Ca^{2+} -dependent membrane binding of annexins as a whole, or individual annexins, requires or is accompanied by conformational changes. Moreover, the structural basis of (possible) membrane insertions of annexins triggered by certain environmental changes like hydrogen ion concentration (see below) need to be described in more detail, possibly also by integrating into such analyses the characterization of folding properties of individual annexin repeats such as the first repeat of annexin A1 (49, 94).

2. Structures of the unique NH_2 -terminal annexin domains and their complexes with protein ligands

Molecular details of the three-dimensional folds of annexin molecules are mostly restricted to the protein core domains (see above) and unique NH_2 -terminal regions of the smaller annexins containing NH_2 -terminal sequences of 16 or fewer residues. In these structures, the NH_2 -terminal sequences extend along the concave side of

the molecule partially engaged in hydrophobic interactions with the protein core. In annexin A3, a direct effect of the NH₂-terminal domain on properties displayed by the core has been shown by replacing Trp-5 (in the unique NH₂-terminal sequence) by alanine. The W5A mutant protein shows a much stronger phospholipid binding, and although having a similar overall structure has a more disordered NH₂-terminal domain. Interestingly, through urea-induced denaturation analysis, it became apparent that the NH₂-terminal domain, even though comprising only 16 residues, unfolds separately from the protein core (128). Thus it appears that the short NH₂-terminal domains of the smaller annexins, located on the concave side of the folded molecule, affect the Ca²⁺-dependent phospholipid binding executed by the convex, or opposite, side possibly through stabilizing or destabilizing slightly different conformations of the molecule. This underscores the regulatory importance of even the small NH₂-terminal domains, a notion that had previously been postulated due to the presence of sites for posttranslational modifications in these regions (see below). Moreover, the finding that subtle differences in the NH₂-terminal sequence, which do not affect the overall structure, result in significantly altered properties could at least in part explain functional diversity among otherwise highly conserved annexins.

Recently, the first complete structure of a longer annexin, annexin A1, has been determined at high resolution (255). Annexin A1 has an NH₂-terminal domain of 40 residues, the first 10–14 of which represent the binding site for a protein ligand of the S100 family, S100A11 (188, 273). Interestingly, in the Ca²⁺-free crystals of annexin A1, this NH₂-terminal sequence forms an amphipathic α -helix and replaces a helix in the tightly packed core domain (helix D of repeat III), which in turn is unwound and partially extrudes from the protein surface (255). Such a structure could have interesting mechanistic and regulatory consequences. Given the tight internal packing of the NH₂-terminal helix, one would assume that this sequence is not available for S100A11 binding in Ca²⁺-free annexin A1. However, upon Ca²⁺-dependent membrane binding, the D helix of repeat III could be forced back into the position described for the Ca²⁺-loaded annexin A1 core (335), thereby freeing the NH₂-terminal helix and enabling this sequence to interact with S100A11. Moreover, such movement could be the prerequisite for the membrane aggregation activity described for annexin A1, e.g., by making accessible a second membrane binding site or a site for homophilic annexin 1 interaction (for a hypothetical model see Fig. 3). Thus, in the case of annexin A1, Ca²⁺ could have a dual regulatory function. First, it could trigger membrane attachment through the convex side of the molecule, and second, by inducing the switch of helix D, it would enable the membrane-bound

protein to interact with cellular protein ligands (S100A11) and/or a second membrane surface. In this respect, it is interesting to note that thermodynamic analyses revealed cooperativity in the binding of Ca²⁺ to annexin A1 (257). Finally, the conformational switch postulated by Rosengarth et al. (255) could also modulate the accessibility of phosphorylatable residues in the NH₂-terminal domain of annexin A1 for their respective kinases (see below), thereby guaranteeing a spatially restricted, probably membrane-dependent, regulation of annexin A1 activities.

The structure of the very same NH₂-terminal domain of annexin A1 comprising residues 1–14 has also been solved in complex with its S100A11 ligand. Cocrystals of S100A11, a homodimeric protein containing two EF hand-type Ca²⁺ binding sites, with the NH₂-terminal annexin A1 peptide revealed a 1:1 stoichiometry, with the two peptides occupying hydrophobic pockets on two opposite sides of the S100A11 dimer (246). The structure of the complex proved to be very similar to that of the NH₂-terminal sequence of annexin A2 bound to a related S100 protein, S100A10 (247). In both annexins (A1 and A2), the first 14 residues form amphipathic α -helices providing the binding sites for two ligands of the S100 protein family (15, 142, 188, 273). At least in the case of annexin A2, it has been shown that the formation of a heterotetrameric complex containing the S100A10 dimer and two annexin A2 chains significantly alters the properties of this annexin *in vitro* and also within cells (for reviews, see Refs. 97, 330). Importantly, the annexin A2-S100A10 complex can aggregate membrane vesicles at micromolar Ca²⁺ levels, a property not shared with monomeric annexin A2 or, as a matter of fact, any other annexin. The structure of the NH₂-terminal annexin A2 peptide in complex with S100A10, in combination with high-resolution images of junctions formed between adjacent membranes by the annexin A2-S100A10 complex, now provides the first detailed structural explanation of this aggregation activity. It appears that due to the highly symmetric nature of the structures, the complex links annexin A2-bound membrane surfaces through the dimerization of S100A10, i.e., the two annexin A2 subunits of the membrane-linking complex are bound to two separate bilayers with the S100A10 dimer connecting them through binding to the NH₂-terminal domains (167, 175, 247). A similar scenario could hold true for the annexin A1-S100A11 complex, which in contrast to annexin A2-S100A10 requires Ca²⁺ binding to the S100 protein and probably Ca²⁺/membrane-bound annexin (see above) for complex formation. Nevertheless, we are still in need of high-resolution structures of complete annexin A2-S100A10 and annexin A1-S100A11 complexes to prove or disprove this attractive model, as well as some evidence that the annexin A1-S100A11 exists *in vivo*.

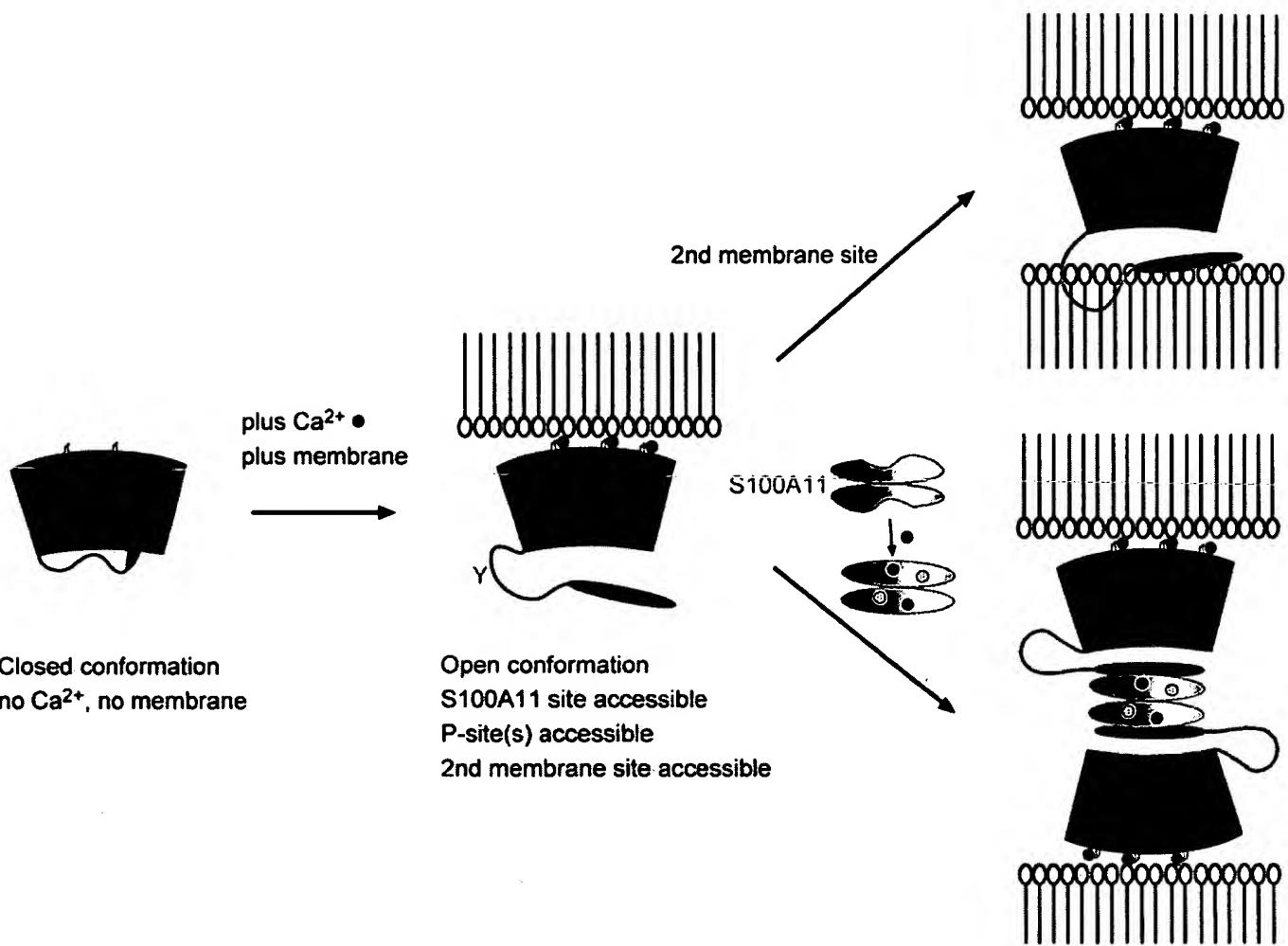


FIG. 3. Model describing the switch of helix D in the annexin A1 structure and its implications for membrane aggregation. In the crystal structure of Ca^{2+} -free annexin A1 (red), the NH_2 -terminal α -helix, which contains the S100A11 binding site (brown), is replacing helix D of the third repeat (255). Ca^{2+} -dependent membrane binding could be accompanied by a conformational change establishing the Ca^{2+} -bound crystal structure of the annexin A1 core (335) and, most likely, a more accessible NH_2 -terminal domain. As a result, the NH_2 -terminal domain can interact with a second membrane surface or the S100A11 dimer, which itself requires Ca^{2+} binding to establish an interaction-competent conformation. An as of yet hypothetical annexin A1/S100A11 heterotetramer would represent an entity capable of linking membrane surfaces (see text and Ref. 255 for details).

B. Annexins as Membrane Binding Proteins: Canonical and Atypical Properties

1. Ca^{2+} -dependent phospholipid binding and vesicle aggregation

Biochemically, annexins are defined as soluble, hydrophilic proteins that bind to negatively charged phospholipids in a Ca^{2+} -dependent manner (they are Ca^{2+} /phospholipid binding proteins). This binding is reversible, and removal of Ca^{2+} by Ca^{2+} chelating agents will lead to a liberation of annexins from the phospholipid matrix. The interaction of annexins with negatively charged phospholipids observed *in vitro* is thought to reflect in a more physiological scenario the binding to cellular membranes, in particular, the cytosolic leaflets of the plasma mem-

brane and various organelle membranes. This canonical annexin property is retained within the annexin cores, the conserved annexin modules most likely representing building blocks designed for peripheral membrane association. However, although Ca^{2+} -dependent phospholipid binding is shared by all annexins, individual members differ significantly in their Ca^{2+} sensitivity and phospholipid headgroup specificity. A large number of reports analyzing the Ca^{2+} -regulated phospholipid binding of annexins *in vitro* have been published, and a comprehensive overview has been given by Raynal and Pollard (244).

Although differences in the binding to phospholipids with different headgroups (e.g., phosphatidic acid, phosphatidylserine, phosphatidylinositol) have long been recognized in *in vitro* studies, it has only recently become

clear that annexin cores also show specificity with respect to their membrane binding in living cells. Through expression of chimeric proteins containing different annexin cores fused to the green fluorescent protein (GFP), it was possible to visualize the distribution of such annexin cores in living cells. Strikingly different distributions were observed within a given cell type showing, e.g., an endosomal localization for the annexin A1 core; an association with certain plasma membrane structures for the annexin A4 core, and a nonmembranous, cytosolic distribution for the annexin A2 core (245). Such live cell experiments have to be extended to reveal annexin dynamics and to circumvent the potential problem that in fixed cells annexin distributions could be subjected to artifacts due to the presence or absence of Ca^{2+} in the fixation/permeabilization buffers. Although the annexin cores carry specificity with respect to membrane binding, an additional layer of such specificity is most likely added by the unique NH_2 -terminal domains of the annexins as in live cells full-length proteins show distributions often differing from the respective cores (73, 196, 245). Moreover, it remains to be seen how interactions with other protein ligands and posttranslational modifications (see below) affect the specific localizations of annexins to certain cellular sites.

Although well described *in vitro*, the physiological importance of Ca^{2+} -dependent phospholipid (and membrane) binding is not understood. However, interesting models have been put forward to assign functions to a peripherally associated and abundant membrane binding protein like, e.g., annexin A5. *In situ*, annexin A5 can form two-dimensional crystals on planar lipid bilayers containing negatively charged phospholipids (26, 229, 248, 249). Such crystalline or semi-crystalline arrangement will most likely affect membrane properties including rigidity, fluidity, and lipid segregation and can therefore participate in the regulation and/or stabilization of membrane domains. Indeed, electron paramagnetic resonance (EPR) spectroscopy reveals that Ca^{2+} -dependent binding of annexin A5 to phospholipid vesicles parallels a rigidification of the membrane (193). Moreover, it was shown that binding of this annexin to the surface of T cells (by an as yet unknown mechanism) delays programmed cell death most likely by generating a certain (in this case extracellular) membrane constraint which in turn interferes with the release of CD4^+ membrane particles (99). On the other hand, membrane binding also affects the annexin protein, as annexin A5, thermodynamically a marginally stable protein (like annexin A1; Refs. 256, 328) is protected to a significant degree from thermal denaturation by Ca^{2+} /phospholipid binding (338).

Annexins are not only capable of binding phospholipid-containing membranes but at least in some cases, e.g., annexins A1, A2, A4, A6 and A7, also mediate membrane vesicle aggregation. Again, phospholipid composi-

tion and Ca^{2+} sensitivity for this aggregation activity differ for individual members (for review, see Ref. 244). As molecular structures of annexins reveal one Ca^{2+} /lipid-binding surface (see above), several models have been put forward to explain an aggregation activity based on the linking of membrane surfaces (see also Ref. 97). One proposal, based on the self-association properties of several annexins, is a protein-protein interaction of annexin molecules bound to two separate membranes (for reviews, see Refs. 51, 244; recent example in Ref. 180). A second explanation is based on the identification of a second membrane binding site in annexin A1 (for review, see Ref. 97 and also discussion in Ref. 23). A sequence in the unique NH_2 -terminal domain of annexin A1, residues 24–35, constitutes a crucial part of this second binding domain and when fused to the core of annexin A5 can confer membrane aggregation activity to this otherwise inactive annexin (40). In contrast to the Ca^{2+} -dependent primary membrane binding via the annexin A1 core, the secondary binding is mainly hydrophobic in nature, and it appears that lateral aggregation of annexin A1 molecules bound to one membrane surface precedes the aggregation mediated through the secondary binding site (24). Interestingly, recent crystal structure determination of full-length annexin A1 suggests that the NH_2 -terminal domain of this annexin only becomes fully accessible when the protein core is linked to a membrane surface via its Ca^{2+} /phospholipid binding sites (255). This could indicate that the second (NH_2 -terminal) membrane binding site in annexin A1 is dormant in the cytosolic protein and only becomes activated when the protein associates with membranes. A third alternative of aggregation activity is probably realized in annexin A6, the only member of the family identified so far with eight instead of four annexin repeats. Here a duplication of the core domain has generated a second Ca^{2+} -dependent phospholipid-binding module, thus allowing for two spatially separated membrane interactions (6). Yet another route is taken by annexin A2 and possibly also other annexins capable of interacting with dimeric protein ligands of the S100 family (see below). The NH_2 -terminal domain of annexin A2 harbors a highly specific binding site for the small dimeric S100 protein S100A10, with protein-protein interaction leading to the formation of a heterotetrameric complex. In this complex, two annexin A2 molecules are noncovalently linked via a S100A10 dimer bound to their NH_2 -terminal domains, thereby generating an entity capable of binding simultaneously to two membrane surfaces through the two annexin A2 cores (175). Thus it appears that although several annexins mediate membrane-membrane contacts, the way this is achieved differs from member to member. This could explain the different Ca^{2+} concentrations required by different annexins for half-maximal vesicle aggregation (for review, see Ref. 244) and also the differing dimensions of annexin-dependent junc-

tions observed in high-resolution cryoelectron microscopy of lipid vesicles aggregated by different annexins in the presence of Ca^{2+} (167).

2. Ca^{2+} -independent lipid binding

Although Ca^{2+} -dependent phospholipid binding remains the criterion of choice for defining an annexin protein biochemically, additional "atypical" lipid binding properties have begun to emerge in recent years. These properties again vary between the different annexins analyzed so far, but it appears that the single most important parameter regulating Ca^{2+} -independent membrane binding is the pH value chosen to analyze the interaction. Annexin A5, for example, binds to and apparently penetrates the bilayer of phosphatidylserine (PS) vesicles at pH 4 (158), and at pH 5 was shown to induce a leakage of PS vesicles (124). Both activities are observed in the absence of Ca^{2+} , whereas at neutral pH Ca^{2+} binding to the protein appears to be a prerequisite for the lipid interaction (158). Most likely, this switch in properties is accompanied by a conformational change in the annexin A5 molecule, which has been shown to occur between pH 4.6 and 4 when the acid-induced unfolding of the protein was analyzed (16). This change is characterized by solvent exposure of a unique tryptophan residue in annexin A5 (Trp-187), and thus is reminiscent of a Ca^{2+} -induced exposure of the same tryptophan at neutral pH (192, 293–296). Conformational changes leading to Ca^{2+} -independent phospholipid binding *in vitro* have also been proposed to occur when Ca^{2+} sites in annexin 2 were inactivated by mutagenesis (79), although such mutations interfere with the intracellular membrane localization of this and other annexins (145, 245).

Considerable progress in analyzing Ca^{2+} -independent annexin-membrane interactions occurring at lower pH has come recently through the introduction of site-directed spin labeling. By engineering protein mutants with unique cysteines and specifically derivatizing these cysteines with a paramagnetic nitroxide side chain, the groups of Haigler, Langen, and Hubbell (168, 169) were able to probe the structure of annexin B12 bound to membranes at lower pH. Combined with the use of reagents that selectively and photoactivatably label amino acid side chains exposed to the hydrophobic domain of the bilayer, they could show that annexin B12 inserts into the bilayer of PS/phosphatidylcholine (PC)-containing vesicles. This insertion is likely to be accompanied by the formation of a continuous transmembrane α -helix. In the solution structure of the molecule, this part forms a helix-loop-helix motif, and it is tempting to speculate that the switch from the helix-loop-helix motif to the transmembrane helix drives a reversible membrane insertion (138, 168, 169). Based on these observations, Langen et al. (168) propose concerted conformational changes in all four

annexin repeats of annexin B12, which are triggered by low pH and involve the formation of several elongated transmembrane helices from helix-loop(turn)-helix structures found in solution (Fig. 4). As a consequence, the entire molecule can assume a transmembrane topology as defined by accessibility to proteases present on either side of the membrane (289). The pH-dependent switch in conformation could be induced by the protonation of certain carboxylate residues found in or close to the loop of the helix-loop-helix motif, which upon deprotonation could drive the protein back to the solution conformation (168). Such a model could also hold true for other annexins, as all have similar solution structures, and its reversibility could perhaps explain why and how certain annexins under certain circumstances can span a lipid bilayer. The latter could be of particular importance in the case of annexins A1 and A2, which also appear to have extracellular activities and for which cell surface receptors have been described (see below). At least in the case of annexins A1 and A6, pH-driven membrane insertion has been identified (103, 258), although it is not clear whether this could lead to membrane translocation.

In addition to the points discussed above, Ca^{2+} -independent membrane associations have also been observed for several annexins at neutral pH. Examples for these types of Ca^{2+} -independent interactions are the association of annexins A2 and A6 with endosomal membranes (120, 145, 160, 275), the binding of annexin A2 to A549 cell membranes (182), and the interaction of annexin A5 with the plasma membrane of platelets (for review, see Ref. 322). At least in part it appears that such interactions are mediated through a binding of the respective annexin to a protein ligand that is itself associated with or embedded in the cellular membrane.

C. Nonlipid Annexin Ligands

1. Annexin complexes with EF hand-type Ca^{2+} binding proteins

The EF hand denotes a helix-loop-helix Ca^{2+} binding motif that is present in a large number of proteins comprising the EF hand superfamily with its distinct subfamilies (for review, see Ref. 154). Several EF hand proteins, in particular those of the S100 subfamily, form complexes with members of the annexin family. S100 proteins are small (~ 10 kDa) proteins characterized by two consecutive EF hands connected by a flexible linker region and flanked by unique NH_2 - and COOH -terminal extensions. Similar to calmodulin, they are thought to interact with and thereby regulate cellular target proteins in a Ca^{2+} -dependent manner (for reviews, see Refs. 68, 267). Three S100 proteins, S100A6, S100A10, and S100A11, were shown to bind specifically to three different annexins, annexins A11, A2, and A1, respectively. The best charac-

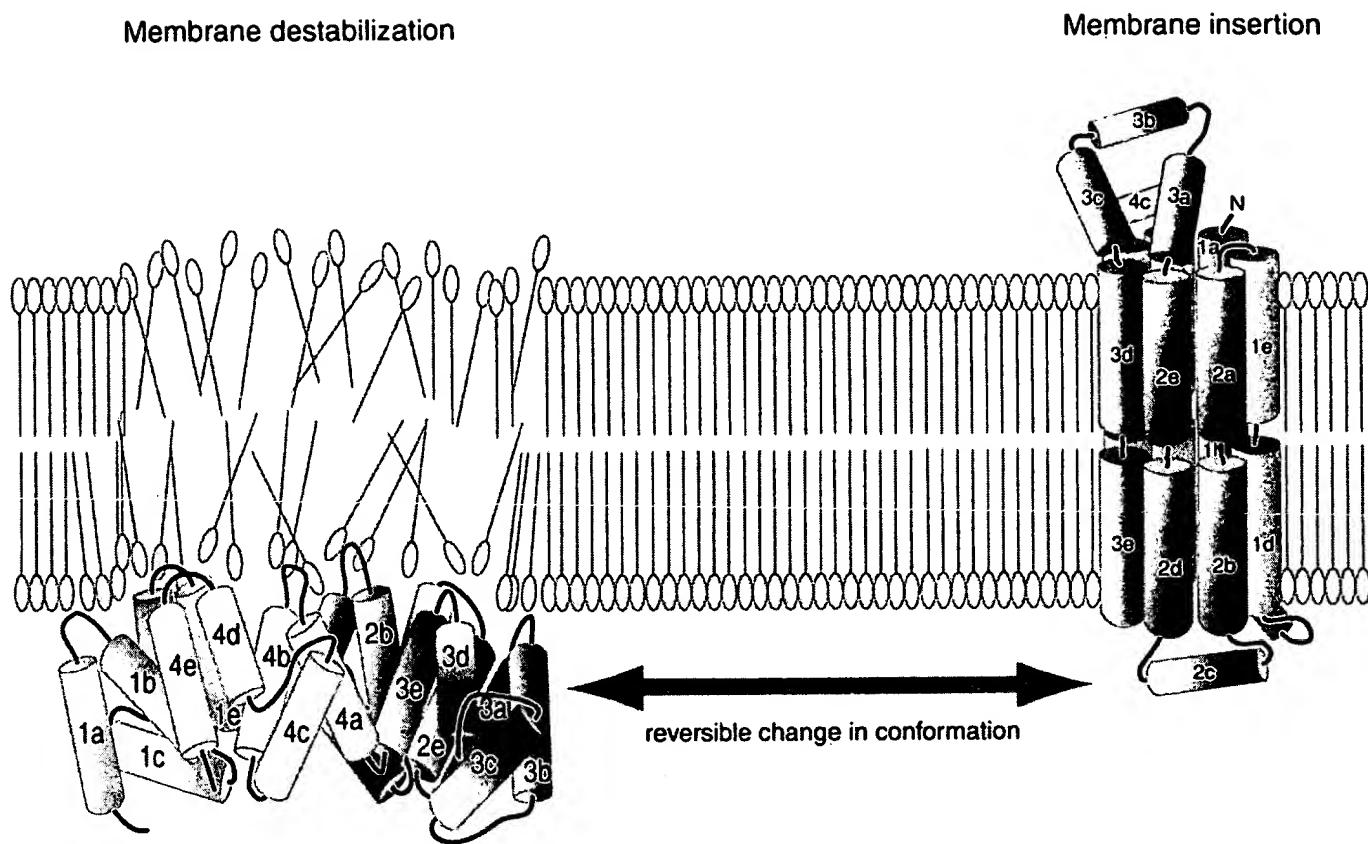


FIG. 4. Peripheral membrane binding and insertion by an annexin. Two potential interaction states for a monomeric annexin molecule with the cytoplasmic leaflet of a hypothetical membrane are shown. The peripherally bound annexin on the *left* assumes the tertiary structure depicted in Figure 2 and has been postulated to increase membrane permeability by apposition of its convex upper surface with the lipid bilayer, which in turn has been suggested to lead to ion flow. The fully membrane integrated structure on the *right* is based on that proposed by Langen and co-workers (168, 169), after protonation at acidic pH, destabilization of the native α -helical structure, and refolding into the seven-transmembrane spanning configuration. Although the proposed structure is obliged to have NH₂ and COOH termini on opposing sides of the bilayer, the orientation shown in the figure is arbitrary.

terized of these annexin-S100 complexes is the annexin A2-S100A10 (p11) heterotetramer. Here it was clearly established that complex formation is highly specific, occurs *in vivo*, can be regulated by posttranslational modifications in the annexin, and modulates properties displayed by the isolated subunits (for review, see Ref. 97). S100A10 is the only member of the S100 family that has suffered deletions and mutations in its two EF hand loops, rendering the Ca²⁺ sites nonfunctional. However, it appears that the resulting conformation of the protein represents a permanently active state with respect to its capacity to bind the annexin A2 target (143, 246, 247). The S100A10 binding site on annexin A2 is restricted to the NH₂-terminal 14 residues and peptides corresponding to this sequence bind to S100A10 with high specificity and affinity. Moreover, such peptides disrupt by competition preformed annexin A2-S100A10 complexes and therefore

can be used as tools for studying complex function (161). At least in studies with synthetic peptides, an important feature of this binding site is the NH₂-terminal acetylation of the NH₂-terminal serine residue of annexin A2, a post-translational modification occurring with high efficiency in eukaryotic cells (15). On the other hand, annexin A2, expressed recombinantly in bacteria and lacking the N-acetyl group, is also capable of binding p11, although the affinity of this interaction has not been compared with that of the acetylated protein (151). The apparent discrepancy in these results remains to be resolved, in particular since only an acetylated NH₂-terminal annexin peptide is capable of disrupting the annexin A2-S100A10 heterotetramer (161).

Complex formation between annexin A1 and S100A11 is based on very similar principles, although in this case Ca²⁺ binding to the S100 protein is required to

establish the interaction-competent form of S100A11 (188, 246, 273). Moreover, it remains to be established if and when this interaction occurs *in vivo*. In contrast to the heterotetrameric annexin A2-S100A10 complexes, standard isolation protocols do not yield annexin A1-S100A11 complexes but only the separated subunits. Likewise, a strong colocalization has not been reported so far, although ectopic expression studies using mutant proteins indicate that annexin A1 can target S100A11 to endosomal membranes in baby hamster kidney (BHK) cells (274). It appears likely that the strict Ca^{2+} dependence of the annexin A1-S100A11 interaction interferes with the visualization or isolation of complexes once Ca^{2+} drops below a certain threshold within the cells or during isolation. On the basis of the high structural similarity of the annexin A2-S100A10 and annexin A1-S100A11 complexes, it is likely that both are heterotetrameric entities with the capacity of linking membrane surfaces in a symmetric manner (see Fig. 3 for annexin A1). While the former complex is known to exist in resting cells irrespective of cellular Ca^{2+} transients (but perhaps regulated by PKC phosphorylation in the NH_2 -terminal sequence of annexin A2; Ref. 144), the latter is probably dependent on (perhaps locally restricted) Ca^{2+} rises and could be of importance during Ca^{2+} -regulated membrane transport events.

The third annexin-S100 protein interaction described to date is that between annexin A11 and S100A6 (312). Although the S100 binding site in annexin A11 is also located in the NH_2 -terminal domain (303, 311), the mode of complex formation is likely to be different. Annexin A11 contains a long NH_2 -terminal domain of almost 200 residues rich in glycine, tyrosine, and proline residues, which resembles that of annexin A7 and possibly lacks a well-ordered three-dimensional fold (177), or contains segments with pro- β -helices (190). However, sequences within the NH_2 -terminal domain of annexin A11 do not resemble the amphipathic helices found in annexins A1 and A2 and thus are unlikely to fit in a homologous manner in a binding pocket formed by the S100 dimer. The physiological consequences of the annexin A11-S100A6 interaction remain to be established, although it is interesting to note that only one NH_2 -terminal splice form of annexin A11 can interact with S100A6 at least *in vitro* (302). Other annexin-S100 interactions have been described, e.g., that of annexin A6 with S100A1 and S100B (96), but the structural basis and physiological significance of such complexes is less well defined. The reader is referred to the S100 literature for further detail (e.g., Ref. 68).

In contrast to S100 proteins, sorcin is a member of the EF hand superfamily containing four and not two of the helix-loop-helix motifs. It binds in a Ca^{2+} -dependent manner to the GYP-rich NH_2 -terminal domain of annexin A7 (28) with the NH_2 -terminal domain of sorcin being required for the interaction (327). Complex formation can

recruit sorcin to the membrane of chromaffin granules, which are a prime site of annexin A7 localization. Moreover, binding of sorcin inhibits the chromaffin granule aggregation mediated by annexin A7 (28), thus underscoring the regulatory importance of complex formation. The common picture emerging from the interaction analyses is that it is most likely to be the annexins that are affected in their properties by EF hand protein binding, rather than the other way round. Although the structural basis of the interaction probably differs between the different complexes, a common feature is the importance of the NH_2 -terminal annexin domain for binding. Protein binding to this unique domain in the respective annexin can have a number of consequences ranging from the establishment of a different physical entity capable of interconnecting membranes (see, for example, Fig. 3) to a protein complex with altered biochemical properties.

2. Annexin interactions with cytoskeletal proteins

A number of annexins have been described as cytoskeleton, in particular F-actin, binding proteins, and it has been suggested that at least some members of the family could participate in regulating membrane-cytoskeleton dynamics. Here we do not survey the entire literature in this area but only focus on recent developments. For an overview of the earlier literature, the reader is referred to previous reviews (97, 244).

Annexin A1 binds to F-actin and also interacts with profilin, a G-actin binding protein and regulator of actin polymerization. Complex formation between annexin A1 and profilin modifies the profilin effect on actin polymerization. Because of the partially overlapping intracellular localization of the two proteins, it is tempting to speculate that the annexin A1-profilin interaction participates in regulating the membrane-associated cytoskeleton (2). In addition to this interaction with a protein involved in actin cytoskeleton dynamics, some colocalization of annexin A1 with tubulin and cytokeratin-8 has also been reported. In A549 human lung adenocarcinoma cells, striking patches of annexin A1 immunolabeling are found at the plasma membrane, which are also positive for these two cytoskeletal proteins (313). It is not clear, however, whether this colocalization reflects a functional interaction.

Annexin A2 is another F-actin binding annexin that also has a Ca^{2+} -dependent filament bundling activity. This bundling activity is particularly pronounced in the case of the heterotetrameric annexin A2-S100A10 complex (for review, see Refs. 97, 330). Recently, the F-actin binding site has been mapped to the COOH terminus of annexin A2, underscoring the specificity of the interaction (80). Annexin A2 is not associated with stress fibers or cytoplasmic actin filaments but appears to play a role in the organization of membrane-associated actin at sites of cho-

lesterol-rich membrane domains. Evidence for this view is severalfold. In the presence of Ca^{2+} , annexin A2 binds to and possibly promotes the lateral association of glycosphingolipid- and cholesterol-rich lipid microdomains (rafts) (8, 119). It has been proposed that in smooth muscle cells this association promotes the binding of annexin A6, which itself mediates the formation of a reversible membrane contact with the actin cytoskeleton (8, 9). On the other hand, annexin A2 could also carry out this task by itself or in conjunction with other actin binding proteins, since cholesterol-sequestering agents specifically release annexin A2 together with the cortical cytoskeletal proteins α -actinin, ezrin, and actin from membranes of BHK and endothelial cells (120; J. König and V. Gerke, unpublished observations). Moreover, expression in epithelial cells of a mutant annexin A2 protein causing the submembrane aggregation of annexin A2 and its ligand S100A10 results in the simultaneous aggregation of a transmembrane raft protein (CD44) and a redirection of actin bundles toward these clusters (216). Thus, due to its Ca^{2+} -dependent membrane and F-actin binding and its intracellular location at sites of membrane rafts, annexin A2 could serve as an organizer of these membrane microdomains and their connection to the actin cytoskeleton.

Annexin A5 has been observed to relocate to the cortical membrane cytoskeleton after activation of platelets. This relocation appears to involve both binding to the plasma membrane and to a specific actin isoform, γ -actin, and is paralleled by an association with the platelet membrane of cytosolic phospholipase A₂, suggesting an interaction between this phospholipase and annexin A5 (319–321). Annexin A6, another actin-binding annexin, has been implicated in mediating in a Ca^{2+} -dependent manner membrane-cytoskeleton contacts in smooth muscle cells (9). Spectrin is another binding partner of annexin A6 in the cortical cytoskeleton. Because annexin A6 promotes a cysteine protease-dependent type of budding of clathrin-coated vesicles at the plasma membrane, it has been proposed that the protein participates in disconnecting the clathrin lattice from the spectrin membrane cytoskeleton during the final stages of coated pit budding (148). With the exception of annexin A2 (98), it is not known whether other annexins share this spectrin binding property. In addition to the family members mentioned above, F-actin binding annexins have recently also been identified in the killifish medaka (297) and in plants (131), although their functional roles in these organisms have not been addressed so far.

3. Other ligands

In addition to Ca^{2+} , phospholipid, EF hand type proteins, and cytoskeleton-associated proteins, a number of other annexin ligands ranging from proteins to RNA and

smaller molecules have been described. An account of such binding partners is given in previous reviews (97, 244), and only the most recent findings are summarized here. Moreover, this section primarily focuses on intracellular binding partners, whereas extracellular protein ligands are discussed when we review extracellular activities of annexins (see sect. IV C).

Annexin protein ligands other than the ones summarized above include the cytosolic phospholipase A₂, which interacts with annexin A1 (156) and the p120 Ras GTPase activating protein (GAP), which through its C2 domain binds to annexin A6 (60). Within annexin A6, the binding site has been mapped to the unique linker region connecting the two four-repeat lobes of the protein (44). This region is not found in other annexins, thus emphasizing the specificity of the interaction. Recently, two protein kinases, Fyn (a src kinase family member) and Pyk2 (a member of the focal adhesion kinase family), have also been found in the annexin A6-p120GAP complex, indicating that annexins could also participate in certain signaling events (43). A link between annexin A6 and signaling has also been inferred from its association with activated PKC- α , which was described in skeletal muscle (272). Another binding partner for annexin A6 was identified in clathrin-coated vesicles isolated from adrenocortical tissue. In a subpopulation of these vesicles, which also contain the transferrin receptor, annexin A6 tightly associates with the GTPase dynamin known to participate in the pinching off of clathrin-coated endocytic vesicles (318). In the vesicle preparations, annexin A2 was shown to bind to a yet unidentified 200-kDa protein, suggesting that these two annexins could participate in defining specific protein-lipid interaction domains during endocytosis (see also below). A similar function has been suggested for annexin A13, which interacts with the C2 domain of the Nedd4 ubiquitin protein ligase, thereby participating in the apical membrane targeting of Nedd4 in polarized epithelial cells (231). Annexin A13 exists in two NH₂-terminal splice variants, a and b, with 13b being specifically targeted to the apical transport vesicles also containing raft components (see below). The binding of annexin A13 to Nedd4 was initially identified in a yeast two-hybrid screen and a number of annexin-protein interactions, e.g., that of annexin A5 with the intracellular domain of the vascular endothelial growth factor (VEGF) receptor Flk-1 (334), have been reported using similar approaches. Interestingly, in all cases reported it has been the protein ligand and never the annexin that was used as the bait in the initial screen.

Some annexins have also been shown to bind to other cellular macromolecules. Annexins A2, A4, A5, A6, and the *Caenorhabditis elegans* protein annexin B7 interact with carbohydrates, in particular glycosaminoglycans, and in some cases the binding sites have been mapped to certain regions within the respective annexin molecule

(83, 139, 152, 159, 265). These interactions are likely to come into play when annexins are present extracellularly, but their functional significance remains to be proven. Nucleic acids comprise yet another class of macromolecules reported to bind to annexins in a Ca^{2+} -dependent manner. Whereas annexin A1 interacts with purine-rich RNA and pyrimidine-rich DNA, annexin A2 has been found associated with mRNA of a distinct polysomal subpopulation (123, 326). It is not known whether and how annexin binding affects stability or functional state of the mRNAs, e.g., in terms of translation efficiency. However, because of the sequence specific binding (A. Vedeler, personal communication) and the fact that annexins A1 and A2 are also actin binding proteins, it has been speculated that the annexin proteins participate in the intracellular positioning of certain mRNAs via an interaction with both the mRNA and the actin cytoskeleton. Single nucleotides binding to certain annexin proteins have also been described in recent reports. While ATP binds to annexins A1 (118) and A6 (for review, see Ref. 11), annexin A7 not only interacts with but also catalyzes the hydrolysis of GTP (34). This latter observation led to the suggestion that annexin A7 acts as an atypical G protein involved in mediating the Ca^{2+} /GTP signal during exocytic membrane fusion (34). However, although GTP and GDP were present in immunoprecipitates of annexin A7 from permeabilized chromaffin cells, the ratio of GTP to GDP was apparently not influenced by Ca^{2+} , raising questions as to how the GTPase activity might be regulated in vivo. Clearly, further work is required to improve our understanding of how annexins interact with nucleotides, especially as annexins lack a consensus nucleotide binding site. In this context, the three-dimensional structures of annexins complexed to nucleotides will be particularly informative, together with the identification of proteins that might modulate any catalytic activity ascribed to annexins, such as the activator proteins, dissociation inhibitors, and exchange factors that collectively regulate other GTPases.

D. Modulation of Annexin Properties by Posttranslational Modifications

Annexins are long known to be targets for posttranslational modifications. In fact, annexin A2 was initially isolated as a major *v-src* protein kinase substrate, and the tyrosine kinase activity of the epidermal growth factor (EGF) receptor has long been known to phosphorylate annexin A1 (see previous reviews, Refs. 51, 97, 111 and also Ref. 259 for an overview). More recently, additional phosphorylations by signal transducing kinases of these and other annexins have been reported with at least some of them affecting annexin properties. Other tyrosine kinases recognizing annexins A1 and A2 as substrates are

those associated with the platelet-derived growth factor (PDGF) receptor, the hepatocyte growth factor/scatter factor, and the insulin receptor (for review, see Ref. 259). In the latter case, annexin A2 only undergoes insulin-triggered tyrosine phosphorylation when receptor internalization is occurring (22). To some extent, this mimics the tyrosine phosphorylation of annexin A1 upon activation and internalization of the EGF receptor and indicates that both annexins and their phosphorylation are mechanistically linked to the internalization/endocytic sorting of certain ligand bound receptors (see also below). Although these phosphorylations are known to occur *in vivo*, their physiological consequences have not been established. *In vitro* or *in situ* studies, however, have revealed alterations in the Ca^{2+} /membrane binding of annexins A1 and A2 phosphorylated at Tyr-20 (by the EGF receptor kinase, Ref. 61) and Tyr-23 (by the src kinase, Ref. 102), respectively. Tyrosine phosphorylated annexin A1 is more susceptible to NH_2 -terminal proteolysis, thus showing altered phospholipid vesicle binding and aggregation activities (for review, see Ref. 111). Moreover, in contrast to the nonphosphorylated form, it requires Ca^{2+} for the association with the membrane of multivesicular endosomes (92). In the case of annexin A2, tyrosine phosphorylation decreases its affinity for phospholipids and interferes with capability of the annexin A2-S100A10 complex to aggregate chromaffin granules at micromolar Ca^{2+} concentrations (132, 236). A mutual influence of Tyr-23 phosphorylation and phospholipid binding is also corroborated by the finding that phosphorylation of annexin A2 by pp60^{src} is significantly enhanced when the protein is bound to PS containing vesicle (17). Recently, annexins A7 and A11 were also described to be phosphorylated on tyrosine residues, in this case in rat vascular smooth muscle cells in response to PDGF. *In vitro* both annexins are also phosphorylated by the Ca^{2+} -dependent tyrosine kinase Pyk-2, the src tyrosine kinase, and the EGF receptor kinase, but the physiological consequences of these phosphorylations have not yet been described (91).

A number of serine/threonine kinases that phosphorylate annexins have also been described. Phosphorylation sites again reside in the unique NH_2 -terminal domains of the annexins, and the modifications in some cases have been shown to affect biochemical properties of the annexins, in particular their affinity for Ca^{2+} /phospholipid (already reviewed in Refs. 97, 244). PKC, for example, has long been known to phosphorylate a number of annexins, with annexin A5 being a remarkable exception as it can serve as a PKC inhibitor (261). The strongest evidence for PKC phosphorylation regulating annexin activities in cells has accumulated in the case of annexin A2 and its involvement in Ca^{2+} -regulated exocytosis in adrenal chromaffin cells. Here, nicotine stimulation leads to annexin A2 phosphorylation by PKC with activation of PKC being a prerequisite for regulated exocytosis (63, 263). A link be-

tween secretion and PKC phosphorylation has also been obtained recently in the case of annexin A7, with PKC phosphorylation activating the Ca^{2+} -dependent membrane fusion displayed by this annexin (33). Other kinases acting on annexins are casein kinase I, which phosphorylates annexin A2, and a yet to be defined histidine-specific kinase which phosphorylates annexin A1 (95, 208). Recent evidence for a participation in intracellular signaling has been obtained for annexin A1, whose expression levels have been coupled to regulation of the extracellular signal-regulated kinase (ERK) pathway in RAW macrophages (1).

As pointed out above, annexin phosphorylation often results in an altered susceptibility toward proteolysis. Although this could be considered a mere indication of a conformational change, it could also reflect an important intracellular consequence of the posttranslational modification directly linked to altered properties displayed by the modified annexin. Cleavage generally occurs in the unique NH_2 -terminal annexin domain with the resulting NH_2 -terminally truncated molecule showing, as revealed in particular for annexins A1 and A2, an altered sensitivity toward Ca^{2+} /phospholipid (for review see Refs. 111, 244) and a different intracellular location (245, 275). In addition, it appears at least in the case of annexin A1 that NH_2 -terminal cleavage by an intracellular protease can occur without prior phosphorylation and can thus itself be considered the regulatory event. This has been shown in human neutrophils where removal by a membrane localized metalloprotease of the NH_2 -terminal eight residues of annexin A1 results in a protein species with a decreased Ca^{2+} requirement for binding to secretory vesicles and the plasma membrane, an event possibly linked to the exocytosis of different vesicle populations (206, 207). In annexin A2, another recently observed modification is a *S*-glutathiolation of Cys-8 in the NH_2 -terminal domain, which is observed after oxidative stress, e.g., in tumor necrosis factor (TNF)- α -treated cells (304). Modification of this cysteine, which is located in the S100A10 binding sequence, does not affect the interaction with S100A10 (143). On the other hand, a general cysteine modification of the annexin A2-S100A10 complex by *N*-ethylmaleimide (NEM), which most likely also affects Cys-8, strongly inhibits the ability of the complex to aggregate lipid vesicles (282). It is not clear whether cysteine residues participate directly in the aggregation activity or whether their derivatization interferes with certain conformational changes in the molecule required for the activity. However, as annexin A2 has been implicated in membrane trafficking events possibly requiring its aggregation activity (see below), NEM, which is frequently used as an inhibitor of membrane fusion, could also affect annexin A2.

Hence, a variety of posttranslational modifications on annexins which also include the *N*-myristoylation of an-

nexin A13a and -b (77, 336) have been described, with most of them affecting the Ca^{2+} and/or membrane binding properties of the molecules and thus their most probable intracellular activity. Future analyses have to reveal how these modifications are mechanistically linked to the different annexin functions.

III. MOLECULAR EVOLUTION OF THE ANNEXIN FAMILY AND REGULATION OF ANNEXIN GENE EXPRESSION

Ever since annexins were first reported in the literature they have been categorized as a structurally conserved family of Ca^{2+} binding proteins. The structural conservation remains a defining characteristic, but the discovery of human annexins A9 and A10 (201, 204) provides what appear to be exceptions to the unifying ability of annexins to bind Ca^{2+} . Nevertheless, the conservation of annexin primary structures extends throughout multicellular eukaryotic species, and the abundance of annexin sequences provides unique insights not only into the evolution of the annexin gene family, but also genetic molecular evolution in a broader sense. For readers seeking detailed accounts of annexin evolution, there are several excellent recent articles (203, 200, 204); here we focus on the major features of annexin phylogeny and ask whether or not functional insight can be gained by examination of molecular relationships between annexins.

A. Molecular Phylogeny of Annexins

Annexins have been described in most eukaryotic organisms, with the exception of those yeasts for which genomic sequences are available. The absence of recognizable annexin-like sequences in *Saccharomyces cerevisiae* had been anticipated by a number of investigators in the field who had used both biochemical and molecular genetic screens in what ultimately proved to be unfruitful searches for yeast annexins. Nevertheless, given the genetic diversity of yeasts, it remains possible that an ancestral eukaryotic annexin will be discovered in certain yeast species.

The simplest organisms known to express annexins are the protist *Giardia lamblia* and the fungus *Neurospora crassa*. The existence of at least three annexins in the protist is surprising given the simplicity of the organism and that more sophisticated multicellular eukaryotes such as *Hydra vulgaris* and *Dictyostelium discoideum* have at most one or two annexins. A long-running question in annexin evolution is whether any of the annexins discovered in these primitive organisms represents the ancestor of the modern vertebrate annexins. Early studies describing the *D. discoideum* annexin as a direct ortholog of vertebrate annexin A7 (106) now appear to be incor-

rect. However, the *D. discoideum* annexin does occupy an interesting niche in annexin evolution. The *D. discoideum* and *N. crassa* annexins share ~40% amino acid sequence identity, which given their evolutionary distance suggests they may be orthologs. Indeed, this annexin has now been discovered in the oyster mushroom and potato fungus (R. Morgan and M. Fernandez, personal communication), suggesting the evolutionary segregation of this annexin to this group of organisms.

A second major group of annexins distinct from the vertebrate cohort have been described in plants (64). Plant annexins are characterized by their lack of variable NH₂-terminal domains and, at least in modern flowering plants, by the absence of type II Ca²⁺ binding sites in repeats 2 and 3. Thus, from an evolutionary viewpoint, plant annexins have evolved in quite distinct ways to those in the animal phyla. The position of the fungal and mold annexins relative to either the animal or plant kingdoms is unclear, but sequence identity of ~40% between *D. discoideum* annexin C1 and human annexin A11 raises the possibility that the former is a direct ancestor of the latter. Analysis of the structure of the annexin A11 gene (10) revealed it to be the common ancestor of up to nine descendent annexins (A1, A2, A3, A4, A5, A6, A8, A9, and A10), indicating that at the time of the early chordate radiation 500–600 million years ago the first vertebrate genomes probably contained the genes for only three annexins, namely, annexins A13, A7, and A11. Exon splicing patterns within the core tetrads of annexins A13, A7, and A11 support the idea that A11 is a descendent of A7 and that this in turn evolved from A13. However, orthologs of annexins A7, A11, and A13 have not been formally identified in any nonvertebrate species, and any direct lineage between annexins in organisms such as *D. discoideum* and vertebrates remains conjectural.

A final point of interest to emerge from studies on the molecular evolution of the annexins concerns the origins of annexin A6. This annexin is unique within the family in that it comprises two of the tetrad repeats found in all other annexins. The two tetrads are joined by a short linking sequence, and it was previously hypothesized that annexin A6 was formed by tandem duplication and fusion of a single tetrad (285, 286). Because the 5'-tetrad of annexin A6 is most closely related to annexin A5, it was proposed that the progenitor of this duplication event was the 5'-tetrad. However, the recent discovery and analysis of the annexin A10 gene provides an alternative and much more persuasive explanation for the origins of annexin A6 (204). First, annexin A10 has greater similarity to the 3'-tetrad of annexin A6 than the two halves of annexin A6 have to one another, and significantly, an unusual single codon deletion near the start of repeat three is present in both annexin A10 and the 3'-tetrad of annexin A6. These and other phylogenetic data suggest that the two four-repeat annexins A5 and A10, which are located on human

chromosome 4q26 and 4q33, respectively, may have duplicated and fused to form the 5'- and 3'-lobes of annexin A6 early in chordate evolution.

Collectively, these phylogenetic studies enable us to put the annexins into a meaningful evolutionary context, but they tell us little about annexin function. Because the invertebrate and plant annexins do not have mammalian orthologs, analysis of annexin function in these simpler organisms may yield little information about the functions of the vertebrate family. Despite the difficulty in extracting functional insight from phylogenetic analysis, the fact that the family of 12 mammalian annexins have been tightly conserved over several 100 million years suggests that these proteins do indeed have important physiological roles.

B. Gene Structures

1. Conservation of genomic structure in the annexins

The structural organization of annexin genes is highly conserved, at least with regard to the positions of intron-exon boundaries (286). Most four-repeat annexins comprise 12–15 exons, the variation depending in large part on the length of the NH₂-terminal domains. Thus annexins A7 and A11 have long NH₂ termini encoded by up to six exons, whereas annexin A5 has a short NH₂ terminus encoded by two exons. For several annexins, particularly those with long NH₂ termini, alternative splicing adds to the diversity of annexin isoforms, which may in turn amplify functional variability within the family as a whole. Annexin A6, which has a duplicated tetrad core and therefore 8 conserved repeats, comprises 26 exons and is the largest annexin gene extending over ~60 kb (285). Within the conserved repeats, the tendency is for intron sizes to be considerably smaller than for those introns that lie between the first two or three exons. In many mammalian annexin genes, the first two or three introns are frequently 10 kb or more, whereas introns within the tetrad core are often <1 kb. Almost all alternative splicing of annexin RNA transcripts occurs within exons that encode the variable NH₂ termini. Given that annexin NH₂ termini contain binding motifs for protein partners and sites for posttranslational modifications, alternative splicing in these domains may contribute to the regulation of annexin function. Perhaps the best-characterized exception to this general rule is the alternative splicing of exon 21 in the seventh repeat of ANXA6. Exclusion of this 18-nucleotide exon gives rise to the characteristic appearance of annexin A6 on gel electrophoresis or Western blotting as a closely spaced polypeptide doublet (205).

Cladistic analysis of the mammalian annexin gene family reveals that annexins fall into three major groups. One group comprises the earliest vertebrate annexins,

these being A7, A11, and A13. A second group includes annexins A4, A5, and A8, and the third group comprises annexins A1, A2, and A3, with annexins A9 and A10 as somewhat distant members. Annexin A6 is more difficult to categorize, because the 5'-tetrad is most closely related to the A4,A5,A8 group and the 3'-tetrad to the A1,A2,A3 group. Nevertheless, the cladistic demarcation of these groups raises the question of whether or not they correspond to functional groupings. Despite the lack of clear functional data for most annexins, it is certainly possible to identify some cohesion within these groups. For example, annexins A1 and A2 both bind proteins of the S100 family, both are physiological substrates for protein serine/threonine and tyrosine kinases, and both are suggested to function in the endocytic pathway. In contrast, annexins A4 and A5 are more closely linked with regulation of ion flow (see sect. IVB), and annexin A6, which arguably belongs in both groups, has been proposed to have roles that impinge on both the endocytic pathway and regulation of Ca^{2+} signaling. Although such notions are purely speculative, the possibility that annexin clades may represent functional groupings might be relevant to the issue of functional redundancy and therefore the design of gene knock-out experiments.

2. Structural and regulatory features

The completion of the human genome sequencing project, together with increasingly sophisticated algorithms for detecting and analyzing DNA sequences, has led to the identification of unusual and interesting elements within certain annexin genes. The most detailed analyses have been conducted for the annexin A5 and A11 genes (10, 137, 251). The rat and mouse annexin A5 genes are unusual in having two promoters. In both species, the promoter proximal to the gene has a high GC content and lacks a TATA box; this is also true for the human and chick annexin A5 genes (48, 76, 227), and all have an abundance of binding sites for the ubiquitous SP1 transcription factor. In contrast, the distal promoter in the rat and mouse annexin A5 genes has a TATA box and conserved binding sites for transcription factors such as AP1, the glucocorticoid receptor, and MyoD. The significance of these observations is not clear, but the possibility exists that under certain conditions, perhaps during cell differentiation, proliferation, or transformation, transcription from the distal promoter results in an annexin A5 transcript that omits exon 2 in which the start methionine is located. Such a transcript would initiate translation within the first conserved repeat, and the protein thus generated would be predicted to lack the NH_2 terminus and have a molecular mass ~ 3 kDa smaller than the full-length protein. Although there are no reports of the natural occurrence of such an annexin A5 splice variant, *in vitro* studies of recombinant annexin A5 showed that a

mutant lacking the NH_2 terminus was unable to mediate a Ca^{2+} influx into phospholipid vesicles (20). Further investigation of these annexin A5 splice forms supported by a clearer understanding of annexin A5 function could reveal the significance of the two promoters for this gene.

The mouse annexin A5 gene also contains an endogenous retrovirus (251) located in intron 4. The MuERV-L sequence is believed to exist in only 100–200 copies in the mouse genome, although there is no evidence that its presence has any impact on the regulation of annexin A5 expression. The same gene also contains a region of Z-DNA (alternating purine-pyrimidine tract) in intron 6, and other Z-DNA sequences have been identified in the annexin A6 (287) and A11 genes (10). Given the abundance of repetitive elements in mammalian genomes, it is not surprising that *Alu* sequences, long interspersed nuclear elements (LINEs), mammalian-wide interspersed repeats (MIRs), and other less common elements have all been described in various annexin genes. For the most part, these appear to be no more than genomic landmarks, but in the case of annexin A6, a LINE-2 element named ALF (for annexin A6 LINE-2 fragment) was shown to function as a potent and highly specific T-cell silencer that may play a role in the downregulation of annexin A6 in T cells exposed to phorbol ester and calcium ionophore (69). This sequence was also shown to be present in other genes including interleukin-4 and PKC- β , both of which are similarly downregulated by this combination of agonists in T cells.

C. Regulation of Gene Expression

Annexins are frequently described as being ubiquitous. This is true in the sense that any single cell type appears to express a range of annexins, or an "annexin fingerprint," but no single annexin is expressed in all cells, implying that regulation of annexin gene expression is tightly controlled. Insight into the mechanisms of annexin gene regulation can be gained by direct investigation of the relevant gene promoters or by indirect analysis of annexin expression.

1. Annexin gene promoters

Relatively few vertebrate annexins have been subjected to detailed promoter analysis. Two annexin A1 genes have been investigated in pigeons, one of which is strongly inducible by prolactin, and both of which bind Y-box factors (237, 323). The promoters for these genes have been partially characterized, but the most detailed analyses have been performed on the human annexin A1 (70, 290), A6 (70), and A7 (301) gene promoters. The annexin A1 gene promoter contains CAAT and TATA boxes that were shown in deletion studies to be essential for minimal promoter activity. Analysis of the annexin A1

promoter also permitted investigation of the sensitivity to dexamethasone, a glucocorticoid analog. Although one study found the promoter to be unresponsive to treatment with dexamethasone (70), the other reported some level of induction (290). The different results may correspond to the use of different cell lines in each report or may reflect the exposure times used which in the former case extended to 8 h, and in the latter to 24 h. Despite these differences, both studies support the notion that annexin A1 is not a glucocorticoid primary response gene. Interestingly, studies on the cytokine responsiveness of the annexin A1 promoter showed the gene to be induced by interleukin-6 (290). This result is consistent with a role for annexin A1 in the acute phase response to inflammation.

The human annexin A6 gene promoter also contains CAAT and TATA boxes, although these are somewhat distal to the transcription start site and in this case the minimal promoter lies downstream of and does not include these elements (70). The most unusual feature of the annexin A6 promoter is a potent T cell-specific silencer located ~600 bases 5' to the transcription start site (69). This element was discussed in section *mB2*. The human annexin A7 gene promoter has also been serially dissected, and it lacks CAAT and TATA boxes but is GC rich and contains many SP1 binding sites (301). The phylogenetically related annexin A11 gene promoter also lacks CAAT and TATA boxes, and it too is GC rich (10). The presence of SP1 binding sites in most, if not all, annexin promoters so far examined, is consistent with their broad patterns of expression, but the existence of other regulatory elements, or in the case of annexin A5 an alternative promoter, suggests that under certain circumstances tight transcriptional control may be exerted.

2. Annexin expression in development and differentiation

The annexin literature contains many reports in which the expression of individual annexins is correlated with cell proliferation, differentiation, or transformation. For the most part, these studies do not reveal any great insight into annexin function, so in this review we focus on instances where annexin expression is developmentally regulated and in which the annexin exhibits a particularly striking and suggestive association with a certain cell type or cellular localization. Many of the clearest examples of potential functional correlates are to be found in the simple eukaryotes. In these cases, the presence of only a few annexins together with fewer cell types allows a more straightforward interpretation of the observations.

Hydra vulgaris expresses at least two annexins, of which annexin B12 (formerly annexin XII) is the best characterized. Annexin B12 was discovered first (270) and is clearly the major annexin in *Hydra*, being expressed at

an estimated 100-fold excess of a second as yet uncharacterized annexin. Immunofluorescence analysis of whole *Hydra* revealed the staining pattern of the two annexins to be segregated, with annexin B12 being largely confined to epithelial battery cells throughout the tentacles, with the second *Hydra* annexin being maximally located in the cytoplasm of nematocytes (269). The epithelial battery cells differentiate from gastric ectodermal epithelial stem cells, whereas nematocytes differentiate from interstitial cells. The battery epithelial cells and nematocytes are closely aligned in *Hydra* tentacles; both are motile and both are actively turned over. The presence of annexins in these cells therefore fits with current models of annexin function in cell matrix adhesion and cell membrane plasticity and remodeling. The nematode worm *C. elegans* is somewhat more complex in that it expresses four annexins (annexins B5 to B8), of which annexin B7 is the best characterized. Annexin B7 (originally *nex-1*) was discovered using classical protein biochemical techniques as a major 32-kDa polypeptide exhibiting reversible Ca^{2+} -dependent binding to phospholipids (53). Immunocytochemical and electron microscopic investigation of this protein revealed it to be associated with a well-defined subset of cell types and structures including the membrane systems of the secretory glands in the pharynx and the uterine wall and vulva. However, the most striking and most intense localization was to the convoluted membranes of the spermathecal valve. These membranes undergo major conformational changes as eggs pass through the valve, suggesting a possible role for annexin B7 in the regulation of membrane fluidity or membrane-membrane and membrane-cytoskeleton interactions. So far, only annexin B7 has been isolated and biochemically characterized, although annexins B6 and B8 (*nex-2* and *nex-3*, respectively) have been shown by RT-PCR to be actively transcribed (57). Of these, annexin B6 has an unusually long NH_2 terminus similar to those that characterize annexins A7 and A11, indicating possible evolutionary linkage with the modern annexins. Although genetic studies have not yet been reported for these annexins, the emergence of RNA interference as a particularly effective technique for preventing gene expression in *C. elegans* (81) opens the way for a detailed and potentially highly informative analysis of annexin function in this organism.

Despite the prevalence of annexins in most eukaryotic species, relatively little is known about developmental regulation of annexin gene expression. A few studies have focused on mammalian annexin gene expression in the developing mouse brain (115, 116), the results of which revealed distinct patterns of temporal and spatial regulation of individual annexins. Other investigators reported developmental regulation of expression of at least four annexins in the loach *Misgurnus fossilis* (140) and the medaka fish *Oryzias latipes* (219). The latter study extended to whole mount RNA *in situ* hybridization to

examine the localization of four annexins during embryogenesis. As with the investigation of nematode annexins, this work identified tightly controlled annexin expression associated with specific organs or cell types. The annexin expressed earliest in medaka embryogenesis is a likely ortholog of annexin A11, which appears transiently in the prechordal mesendoderm and hindbrain. The three other medaka annexins, which may be orthologs of annexins A1, A4, and A5, all appear later in embryogenesis but in a range of tissues including liver, floor plate, and skin.

These studies represent only a small part of a large and fragmentary literature relating to the developmental, cell growth, and differentiation-dependent regulation of annexin expression. The picture that emerges from these studies is that for many annexins expression patterns are broad, which might imply fundamental roles in cell physiology for most members of the family. For the majority of annexins, functions suggested on the basis of observations made in a single cell type may therefore be incorrect. However, other annexins are undoubtedly restricted in their patterns of expression, sometimes with regard to cell and tissue development and sometimes in terminally differentiated cells. In these cases, exemplified by annexin A13, which is clearly involved in apical vesicle transport in certain polarized epithelial cells, it is more reasonable to predict a specialized cell type-specific function. In simpler eukaryotes with fewer annexins, of which several examples have been described here, there is now the prospect of combining genetics with developmental analysis to provide new information about function. Even if the absence of orthologous annexins in vertebrates presents a bar to direct extrapolation of function, the knowledge will impinge on the experimental design of genetic approaches to annexin function in species such as the mouse.

IV. FUNCTIONAL DIVERSITY WITHIN THE ANNEXIN FAMILY

A. Annexins in Membrane Traffic and Organization

A priori, annexins are intracellular proteins, and their denominating property, i.e., binding in a Ca^{2+} -regulated manner to negatively charged phospholipid surfaces, strongly argues for their functioning in conjunction with such phospholipids that are enriched in the cytoplasmic leaflets of cellular membranes. Exceptions are, however, disturbed cells undergoing apoptosis which display negatively charged phospholipids on their surface. In fact, it is the canonical annexin property of Ca^{2+} -dependent binding to acidic phospholipids which led to the introduction of annexin A5 as a diagnostic tool for labeling the surface of apoptotic cells. It has to be emphasized here that this diagnostic binding, albeit very useful, does not necessar-

ily have any implications for the in vivo function of annexin A5.

A large number of reports have provided circumstantial and also more direct evidence for annexins functioning in intracellular membrane organization. These include the detailed analyses of annexin distributions within different types of mostly cultured cells. A survey of these localization studies has been presented before (97). As a whole it appears that different annexins show strikingly different subcellular distributions and often reside in both a cytosolic and a membrane-associated pool, with a switch between the two typically being regulated by Ca^{2+} . The respective target membranes identified for different annexins are in most cases the plasma membrane and membranes of the biosynthetic or the endocytic pathway, and it has therefore been concluded that annexins function in these membrane trafficking steps.

More recently, studies on the intracellular location of annexins have been extended to live cells in approaches using GFP fusions. Such analyses revealed that annexin A1 associates with membranes of the endosomal, transferrin-accessible system of HeLa cells in a manner dependent on active Ca^{2+} binding sites being present in the protein. Moreover, it was shown that removal of the unique NH_2 -terminal domain of the protein results in a change in intracellular localization with the annexin A1-core being targeted to late endosomal membranes. Interestingly, this also appears to be specific since protein cores of other annexins (annexins A2 and A4) show different distributions in live HeLa cells (245). In light of the finding that proteolytic cleavage within the NH_2 -terminal domain of annexin A1 is likely to occur within cells (possibly triggered by phosphorylation, see above), the distinct localizations of full-length and NH_2 -terminally truncated annexin A1 could reflect distinct functions of the two species. Full-length annexin A2 localizes to the plasma membrane in living HeLa and HepG2 cells and also to membranes of the endosomal system in living BHK and rat basophilic leukemia cells (196, 245, 342). In the latter case, inspection by evanescent field microscopy of pinocytic vesicles formed under mildly hyperosmotic conditions revealed the presence of annexin A2-GFP in actin tails propelling these pinosomes. Moreover, formation of such pinocytic rockets is inhibited by overexpression of a mutant protein dominantly interfering with the annexin A2 localization, thus suggesting an important role of this annexin in organizing interfaces between certain membranes or membrane domains and the actin cytoskeleton (196). An annexin A7-GFP chimera was also localized recently to discrete intracellular structures, in this case in differentiated myoblasts (45). Upon subcellular fractionation, the annexin A7-containing membranes copurify with caveolin-3, but a role of annexin A7 in, e.g., the establishment or stabilization of t tubules during myogen-

esis, remains to be shown and is not as yet evident in annexin A7-deficient mice (see sect. IVD).

1. Annexins in the biosynthetic pathway

A number of annexin proteins, including annexins A1, A2, A3, A6, A7, A11, A13, and B7, have been linked to exocytotic processes, more specifically post-*trans*-Golgi network events in the biosynthetic pathway (for reviews, see Refs. 30, 51, 97, 244). The most compelling evidence for such an involvement which go beyond the mere localization of the protein to secretory organelle membranes and/or the plasma membrane has been reported for annexins A2 and A13. Annexin A2 has been identified as a cytosolic protein that can retard the rundown of secretory responsiveness to Ca^{2+} stimulation of permeabilized chromaffin cells when added exogenously as a purified protein. In this assay, the annexin A2-S100A10 complex, which is localized in several cell types to the sites of plasma membrane/secretory granule membrane contact and/or intergranule contact (212, 276, 277) and which is capable of aggregating vesicles (see above), is more efficient than the monomeric annexin protein. Moreover, PKC phosphorylation of annexin A2 is required for the activity (263) (recall that PKC is activated upon nicotinic stimulation of chromaffin cells), and a peptide corresponding to an NH₂-terminal annexin A2 sequence containing the PKC site inhibits catecholamine secretion in nicotine-stimulated chromaffin cells. Interestingly, it appears that the function of the annexin A2-S100A10 complex in chromaffin granule exocytosis is restricted to adrenergic cells as the S100A10 subunit is not expressed in the noradrenergic cell type (38) (for review, see Ref. 5). By correlation, it was also inferred that the annexin A2-S100A10 complex participates in lung surfactant secretion from alveolar type II cells as phenothiazines inhibited this secretion in a manner similar to their inhibition of annexin A2-S100A10-mediated vesicle aggregation (181). Yet another Ca^{2+} -triggered exocytosis event is the regulated secretion of different granule contents from endothelial cells, the most prominent being the von Willebrand factor stored in Weibel-Palade bodies. With the use of a whole cell patch-clamp approach combined with membrane capacitance recordings, it was shown that disruption of the annexin A2-S100A10 complex by a competitor peptide corresponding to the S100A10 binding site on annexin A2 markedly inhibits the Ca^{2+} -dependent exocytotic membrane fusion (161). Annexin A2 and S100A10 in endothelial cells are located at the plasma membrane and not found on Weibel-Palade bodies. Hence, it has been proposed that the complex indirectly functions in endothelial granule exocytosis by organizing the plasma membrane in a manner supporting the granule-plasma membrane fusion event (161, 213). In line with this proposal, annexin A2 is found concentrated at certain subdomains

of the plasma membrane and seems to provide a link between such subdomains and the actin cytoskeleton (see below).

Annexin A13, a myristoylated member of the family occurring in two NH₂-terminal splice variants (a and b), is only expressed in a limited subset of polarized epithelial cells. Here, the 13b variant is localized specifically to the TGN, post-TGN carrier vesicles, and the apical plasma membrane. Antibodies directed against the unique exon encoded sequence in the annexin A13b splice form interfere with carrier vesicle transport to the apical but not the basolateral membrane domain of permeabilized Madin-Darby canine kidney (MDCK) cells, suggesting a very specific role of the protein in this transport step (77). Annexin A13b binds to sphingolipid- and cholesterol-rich domains (rafts) that bud off the TGN and that are destined for the apical plasma membrane in a transport step requiring a microtubule minus end-directed motor (214). The TGN budding is inhibited by annexin A13b antibodies and stimulated by myristoylated but not unmyristoylated annexin A13b (165). The other splice variant, annexin A13a, also stimulates apical biosynthetic transport but, in contrast to annexin A13b, also appears to be involved in the basolateral delivery in polarized cells (172). Due to their specific and in some cases regulated association with raft domains, the involvement of annexin A13 isoforms in post-TGN transport to the plasma membrane could be based on a membrane-organizing effect and thus could mirror the situation discussed for annexin A2 in Ca^{2+} -regulated exocytosis in endothelial cells. Thus the membrane-organizing capacity of annexins, which differs in extent, regulation, and target membrane between different members of the family, could represent the mechanistic basis of annexin effects in membrane transport events.

Other biosynthetic membrane transport steps affected by annexins include Ca^{2+} -dependent secretion in neutrophils, which in a streptolysin-O (SLO)-permeabilized cell system is stimulated by annexins A1 and A3 (254), and Ca^{2+} -induced insulin secretion in pancreatic β -cells, which in SLO-permeabilized cells is inhibited by annexin A11 antibodies (135). However, in all cases, live cell experiments, e.g., the injection of antibodies or interfering peptides/proteins or the generation of cells deficient in specific annexins combined with a subsequent characterization of transport steps in the modified cells, are required to corroborate and more specifically define the role of annexins in exocytosis.

2. Annexins in the endocytic pathway

Membranes of the endosomal system have also been identified as target structures for several annexins, and the mode of membrane binding has been studied extensively, both in terms of the specific target membrane

selected by the individual annexins and in terms of the structural requirements for membrane binding within the annexin molecule. Some of the literature describing annexin-endosome interactions has been reviewed before (97, 108). More recently, the specificity and thus most likely functional importance of such interactions has received support by a number of observations regarding annexins A1, A2, and A6. Live cell experiments analyzing the intracellular distribution of annexin-GFP chimeras have underscored the importance of the unique NH₂-terminal domain in positioning the individual annexin at certain target membranes (see above). Annexin A1, for example, is found on early, transferrin-accessible endosomes in BHK and HeLa cells, although some protein is also present on multivesicular endosomes, at least in mouse fibroblasts (92, 245, 275). Upon removal of the NH₂-terminal domain, the resulting annexin 1 core domain redistributes to late endosomes with the interaction still being Ca²⁺ dependent and specific, i.e., not observed with other highly homologous annexin cores (245, 275). This switch could also occur under certain cellular con-

ditions, e.g., when upon internalization of the EGF receptor annexin A1 becomes phosphorylated on Tyr-20 and thus more susceptible to NH₂-terminal proteolysis (see above). The proteolysis removes at least part of the NH₂-terminal domain and thus not only the sequence required for localizing the protein to early endosomes but also the binding site for the annexin A1 ligand S100A11. As a result, a putative heterotetrameric annexin A1-S100A11 complex capable of linking membrane surfaces would be disrupted. Together with the finding that the association of annexin 1 with multivesicular endosomes is regulated through phosphorylation by internalized EGF receptors, the proposed role of the protein in mediating the inward vesiculation in multivesicular endosomes (92) could be envisaged as depicted in Figure 5. Annexin A1 in its Ca²⁺-regulated complex with S100A11 (which is targeted to early endosomes by annexin A1, Ref. 274) could organize the limiting membrane of multivesicular endosomes *in situ nascendi*, i.e., early endosomes or budding endosomal carrier vesicles in the process of becoming multivesicular, in a way that supports the inward vesiculation.

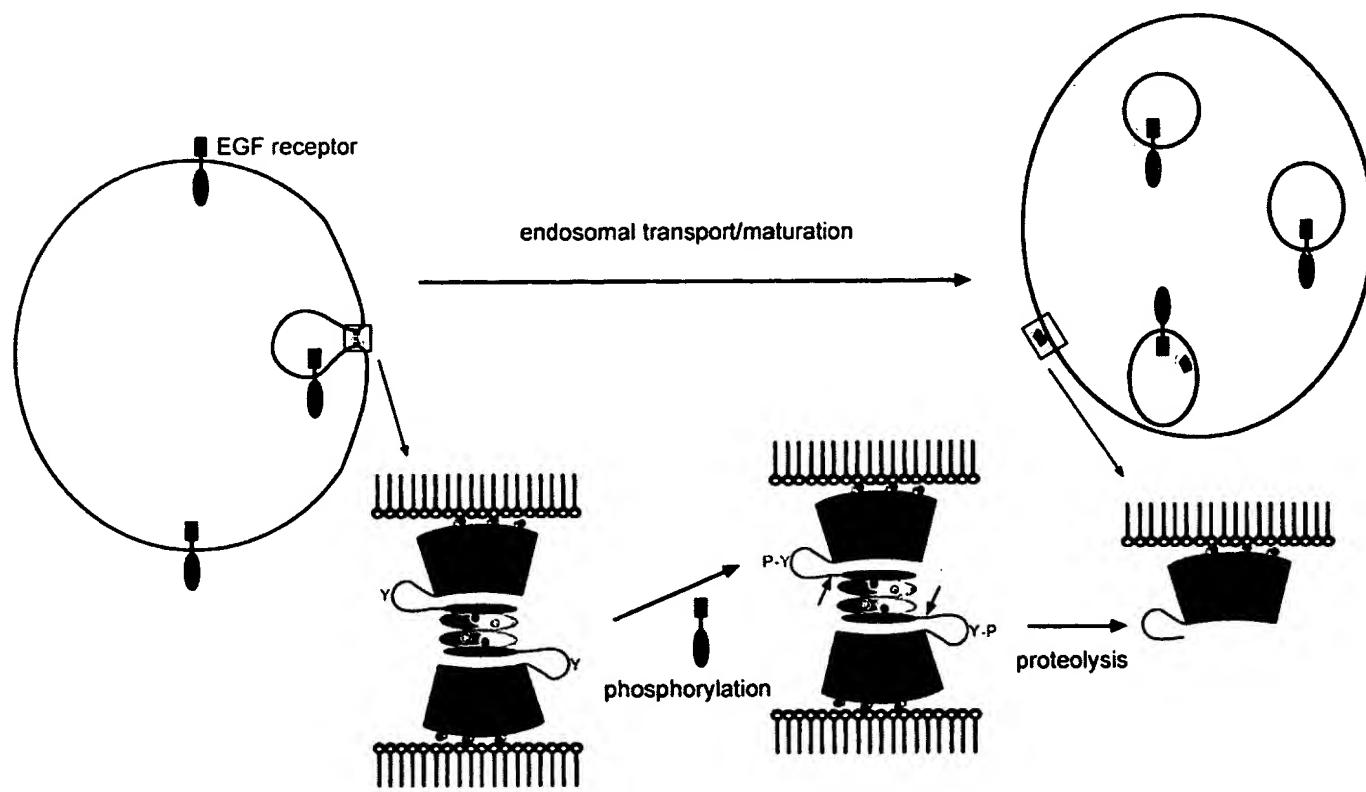


FIG. 5. Model describing a potential participation of annexin A1-S100A11 in the inward vesiculation process generating multivesicular endosomes. Owing to its potential membrane linking properties, the as of yet hypothetical heterotetramer of annexin A1 (red) and S100A11 (orange) could stabilize membrane interactions required for inward budding. Phosphorylation of Tyr-20 of annexin A1 by the internalized epidermal growth factor (EGF) receptor (92) renders the protein more susceptible for proteolysis occurring in the NH₂-terminal domain. Proteolytic cleavage would release the S100A11 dimer (together with the NH₂-terminal annexin A1 sequence) in a process that could accompany the actual membrane fission and internal vesicle release. As a result, an NH₂-terminal foreshortened annexin A1 would be present on multivesicular/late endosomal membranes, a localization in line with that of ectopically expressed annexin A1 core-green fluorescent protein (245).

Once the inward budding is about to be completed with the final fusion process, annexin A1 becomes phosphorylated by internalized receptors destined for degradation. This leads to limited proteolysis and thus disruption of the complex between annexin 1 and S100A11 in a process which might be coupled to the actual fusion. This activity might not be essential for endosome maturation/transport to occur but might facilitate the kinetics by providing a supporting membrane scaffold. Whether and how this model relates to observed stimulatory effects of annexin A1 on cell proliferation, EGF receptor synthesis, and phosphorylation remains to be established (62).

Annexin A2 was also identified on early endosomes (in addition to its localization at the plasma membrane and on certain secretory granules). Interestingly, its interaction with endosomal membranes occurs in the absence of Ca^{2+} and is primarily mediated by the NH_2 -terminal domain, which upon fusion to an annexin A1 core can transfer this unique property to the otherwise Ca^{2+} -sensitive annexin A1 (120, 145, 160). It is not clear whether the NH_2 -terminal annexin A2 sequence in question interacts with a protein receptor, e.g., a 200-kDa protein identified in ligand blotting experiments in certain clathrin-coated vesicle preparations (318), or specifically associates with a unique lipid structure, e.g., cholesterol-rich membrane domains that are required for this atypical annexin-membrane interaction (120, 160). Annexin A2 is not distributed evenly over the early endosomal compartment. Immunoaffinity approaches reveal that it is enriched on Rab11- (and also transferrin receptor) but not Rab5-positive endosomes of Chinese hamster ovary (CHO) cells, whereas it is not found in transferrin receptor-positive recycling endosomes of polarized MDCK cells (93, 315). In BHK cells, on the other hand, endosomes isolated on immobilized annexin A2 antibodies are positive for the transferrin receptor but not the early endosomal antigen EEA1. This correlates with the ultrastructural colocalization of annexin A2 and the transferrin receptor on endosomes of BHK cells (341). Thus it appears that the fine tuning of annexin A2 localization within the early endosomal compartment is handled differently in different cells, possibly in conjunction with its proposed function as a scaffolding protein organizing and/or stabilizing rafts (see below).

A similar scenario could hold true for annexin A6, which has been localized to different endosomal membranes in different cells. In rat liver hepatocytes, it is found on an apical endocytic compartment, whereas it is present on late endosomes/prelysosomes of NRK fibroblasts (218, 235). Furthermore, some annexin A6 is also associated with the plasma membrane and clathrin-coated vesicles (179, 318), where it could function in facilitating coated pit budding. Earlier experiments employing immobilized plasma membranes and taking the loss of clathrin from such membranes as a measure for

coated pit budding reported an inhibitory effect when the cytosol required in this Ca^{2+} - and ATP-dependent reaction was depleted of annexin A6 (179). More recently, with the use of the same assay, it was shown that annexin A6 is only required for a certain type of coated pit budding, one sensitive to the cysteine protease inhibitor ALLN. The involvement of annexin A6 depended on its association with spectrin indicative of a function of the protein in remodeling the spectrin lattice as a prerequisite for efficient budding (148). Moreover, the same study revealed that microinjection of a truncated annexin A6 mutant inhibited low-density lipoprotein (LDL) uptake, thus paralleling the inhibitory effect of the mutant on coated pit budding in the *in vitro* assay. Other coated pit budding events (those not affected by ALLN) do not require annexin A6, thus providing a possible explanation for the finding that receptor-mediated endocytosis in annexin A6-negative A 431 cells is not stimulated by ectopic overexpression of the protein (288). On the other hand, ectopic overexpression of annexin A6 in CHO cells has a stimulatory effect on LDL receptor endocytosis, however, only when the receptor itself is overexpressed as well. In these experiments, annexin A6 remained associated with LDL-containing vesicles also at later stages of the endocytic pathway, possibly suggesting additional functions of the protein in late endocytic events (107). Such functions are likely to include trafficking events leading to LDL degradation, since microinjection of a mutated annexin A6 into NRK cells causes a retention of LDL in the prelysosomal compartment (234).

3. Annexins and phagocytosis

A translocation of different annexins to the membrane of maturing phagosomes has been observed in a number of phagocytic cells and has been taken as an indication for the involvement of the proteins in phagocytosis (previously reviewed in Ref. 97). Such observations have been extended in the recent past showing, for example, that annexins A1–A5 are present on isolated phagosomes from J774 macrophages, with the levels of annexin A4 (but not those of the other annexins) increasing with age of the maturing phagosomes (66). In another study using macrophage-like cells and analyzing Fc receptor-mediated phagocytosis, annexins A7 and A11 in addition to the members mentioned above were found to translocate to the phagosomes upon particle ingestion (230). In differentiated human monocytes, phagocytic uptake of *Brucella* bacteria (opsonized or nonopsonized) is accompanied by a recruitment of annexin A1-positive structures to the site of entry (164), whereas neutrophils phagocytosing yeast cells show a translocation of annexin A11 to the periphagosomal region (284). When the same cells take up an attenuated strain of *Mycobacterium tuberculosis*, the intracellular distribution of annexins A1

and A5 remains unchanged while annexins A3, A4, and A6 are translocated from the cytoplasm to the proximity of the bacteria containing phagosomes. In the case of annexin A4, this even occurs in Ca^{2+} -depleted neutrophils (189). Similar observations have also been made in human dendritic cells phagocytosing *M. tuberculosis* (170). Although these reports point to a role of the respective annexins in phagosome maturation, e.g., by facilitating certain transport or phagosome-endosome fusion steps, they so far only represent circumstantial evidence. We are still in need of functional approaches, e.g., analysis of phagocytosis in cells with an altered annexin expression or in the presence of dominantly interfering compounds, to make a conclusive connection between annexins and phagocytosis.

4. Annexins and the establishment/stabilization of membrane domains

Recent years have seen a formulation of the concept of membrane microdomains (rafts) being involved in aspects of membrane transport and also representing platforms for signaling events (for review, see Refs. 27, 281). Rafts are lateral assemblies of membrane patches rich in sphingolipids and cholesterol, which due to their high content of saturated hydrocarbon chains form a liquid-ordered phase in the more disordered background of glycerolipids containing unsaturated fatty acid chains. Biochemically, rafts (or detergent-resistant membrane complexes, DRMs) are defined by their resistance toward treatment with nonionic detergent in the cold and their ability to float to low density in sucrose density gradients. A number of membrane proteins have been described as raft associated. Typically, these proteins contain a certain fatty acid, isoprene or lipid moiety (e.g., glycosylphosphatidylinositol or GPI) which helps anchor them in the membrane raft. Most of the typical lipid and also protein components of rafts described to date are those found in the exoplasmic leaflet of the bilayer. The inner leaflet of these microdomains is less well characterized, although it appears to contain glycerophospholipids with a higher degree of saturation than the total plasma membrane (88) and also is likely to be enriched in cholesterol (281). Moreover, information on proteins associating specifically with the cytoplasmic side of rafts and thereby possibly regulating their assembly and dynamics is rather scarce. It is here where annexins could come into play as several members of the family have been identified in raft preparations and seem to associate with rafts in a manner which is in some cases but not strictly regulated by Ca^{2+} . Recent evidence in support of this view is summarized below for different annexins.

Rafts and membrane microdomains are not restricted to the plasma membrane but are also found in membranes of the biosynthetic (Golgi) and the endoso-

mal system (209, 279, 280). Annexin A1, a substrate of the EGF receptor kinase implicated in endosomal sorting of the receptor (see above), is also a substrate of PKC and localizes with the active enzyme to the endosomal compartment. After PKC activation initiated by exposure of cells to phorbol esters, downregulation of the enzyme appears to occur via translocation from the plasma membrane to endosomes, a process which is inhibited by caveolin binding drugs. Because PKC translocation and thus its colocalization with annexin A1 does not depend on the endosomal GTPase Rab5, it has been proposed that the transport is mediated through caveolae, cell surface invaginations containing a subset of lipid rafts which are formed by polymerization of the cholesterol-binding caveolin proteins (238; for review on caveolae, see Refs. 3, 136). Thus it appears that annexin A1 could also be involved in the downregulation of membrane-bound PKC through caveolae-mediated traffic to endosomes. Although evidence in support of this hypothesis is vague and circumstantial, several lines of research have strongly and more directly implicated annexin A2 in the organization and dynamics of membrane rafts. In several types of cells, annexin A2 associates with membrane rafts (biochemically defined as described above) in both a Ca^{2+} -dependent and a Ca^{2+} -independent manner. These include adrenal chromaffin cells where, after nicotine stimulation and in the presence of 1 μM Ca^{2+} , the protein translocates to Triton X-100 (TX-100)-insoluble membrane subdomains (262) as well as MDCK, polarized mammary epithelial, and smooth muscle cells where raft association is also Ca^{2+} dependent (8, 119, 216). On the other hand, BHK as well as bovine endothelial cells contain annexin A2, whose association with rafts is not sensitive to Ca^{2+} chelation but correlates with the amount of membrane cholesterol (50, 120). Interestingly, annexin A2 associated Ca^{2+} independently with BHK or endothelial cell membranes can be specifically released together with a subset of cortical cytoskeletal elements (actin, α -actinin, ezrin, and moesin) by sequestration of membrane cholesterol, suggesting a link between raft-associated annexin A2 and the membrane underlying actin cytoskeleton (120; König and Gerke, unpublished observations).

A role of annexin A2 as an organizer of membrane domains has gained further support in the case of the Ca^{2+} -dependently raft-associated protein. In the sarcolemma of smooth muscle cells, the dynamics of rafts, their lateral assembly, and association with the actin cytoskeleton appear to be regulated by changes in intracellular Ca^{2+} concentrations occurring during smooth muscle contraction. These changes correlate with the Ca^{2+} -dependent association of annexin A2 with membrane rafts and the translocation of annexin A6 to a membrane-cytoskeleton complex. Hence, it was proposed that an initial Ca^{2+} rise in smooth muscle cells triggers the binding of annexin A2 to lipid rafts and a clustering of these rafts

which is promoted by lateral annexin assembly. As a consequence, the spatial organization of, e.g., membrane receptors, is altered leading to a second Ca^{2+} transient further elevating intracellular Ca^{2+} . This is proposed to trigger a translocation of annexin A6 to the sarcolemma where it could be involved in the formation of bonds between the plasma membrane and the actin cytoskeleton (8). Annexin A2 is a F-actin binding protein itself (98) and thus could also participate more directly in the formation of membrane-cytoskeleton links. In a recent study it was shown to colocalize in basolateral lipid rafts of mammary epithelial cells with the hyaluronic acid receptor CD44. Antibody-induced clustering of CD44 leads to a similar clustering of annexin A2. Even more interestingly, clustering of annexin A2 at the cytoplasmic side of the membrane, which was achieved through ectopic expression of a *trans*-dominant annexin A2 mutant protein, led to the enrichment of CD44 in the annexin A2-positive patches. Moreover, a reorientation of F-actin toward the annexin A2 clusters was observed (216). A rearrangement of cortical actin can also be induced by certain pathogens, e.g., enteropathogenic *Escherichia coli* (EPEC), which induce the formation of actin-rich pedestals underneath their site of host cell attachment. Interestingly, bacterial attachment triggers a clustering of membrane raft components and a recruitment of annexin A2 to the attachment sites, suggesting that annexin A2 could participate in this process by stabilizing raft patches and their linkage to the actin cytoskeleton beneath adhering EPEC (343).

Collectively, these findings indicate that annexin A2 could represent a cytoplasmic protein peripherally associating with the cytoplasmic leaflet of membrane rafts, thereby stabilizing these domains and providing a link with the cortical actin cytoskeleton. Such a function would depend crucially on the membrane association of annexin A2 and therefore could be regulated in two ways, by membrane cholesterol content and local Ca^{2+} concentration, as indeed shown recently for annexin A2 associated with endosomal membranes (279, 341) or artificial liposomes containing cholesterol (7). This dual regulation mode reflects itself in the structure of the molecule as the NH_2 -terminal domain mediates a Ca^{2+} -independent interaction with cholesterol-rich membrane domains (160) and the COOH -terminal protein core harbors the Ca^{2+} -regulated binding site. As depicted in Figure 6, this could mean that some annexin A2 binds to cholesterol-containing membrane domains or specific receptors therein, already in the absence of Ca^{2+} . Once Ca^{2+} rises, e.g., during regulated exocytosis, at the sarcolemma upon smooth muscle cell activation or during certain types of endosomal fusion (129), additional annexin A2 molecules are recruited to the same sites providing a sort of membrane scaffold and, given they reside in complex with the S100A10 dimer, contact sites to other membranes or the cytoskeleton. In this view the annexin serves a more

structural role in the membrane periphery affecting indirectly a number of membrane transport events, thus functioning in a manner similar to that discussed for membrane skeleton proteins, e.g., spectrin or spectrin-associated proteins present on intracellular membranes (14, 197, 340).

As already mentioned, annexin A6 has also been implicated in the organization of membrane domains, in particular their association with the cytoskeleton in smooth muscle cells (8, 9). It could serve a similar function in mammary epithelial cells as a fraction of annexin A6 is recovered from the TX-100-insoluble fraction from these cells in a manner which appears to be regulated during polarization of the cells (171). Moreover, annexin A6 associates with raft fractions from synaptic plasma membranes in a Ca^{2+} -dependent manner (217). Whether and how this relates to the spectrin binding of annexin A6 and its proposed function in clathrin-coated pit budding remains to be shown. The sole annexin whose association with lipid rafts was shown to be functionally important for membrane trafficking events is annexin A13b (see also above). Together with its NH_2 -terminal splice variant, annexin A13a, it is the only member of the family that can be NH_2 -terminally myristoylated (77, 336). Annexin A13b is located in the apical compartment of polarized MDCK cells and found on the *trans*-Golgi network, at the apical cell surface and on exocytic apical carrier vesicles whose formation is inhibited by anti-annexin A13b antibody (165). As judged by several criteria including flotation in Optiprep gradients of TX-100-insoluble fractions obtained from apical carrier vesicles, annexin A13b clearly is a raft-associated protein, and it was proposed to function by binding to apical rafts that bud off the *trans*-Golgi network (165). Recently, it was also shown that annexin A13b participates in mediating the apical membrane targeting of the ubiquitin ligase Nedd4. The enzyme contains a C2 domain and associates with lipid rafts in a Ca^{2+} -dependent manner, most likely through an interaction of this Ca^{2+} -sensitive C2 domain with the raft-associating annexin A13b (231). Annexin A13a differs from the 13b variant by a deletion of 41 amino acid residues from the unique NH_2 -terminal domain and a somewhat broader intracellular distribution as it is also found at the basolateral membrane. Interestingly, its association with lipid rafts differs between the apical and the basolateral compartment of polarized epithelial cells with only the latter requiring Ca^{2+} . Moreover, and in contrast to annexin A13b, the 13a variant appears to be involved in the basolateral transport route (172). Thus different annexins seem to participate to differing extents in the organization of membrane domains (e.g., lipid rafts) with their association with these domains and thus their role in the process being regulated by changes in cytosol conditions (Ca^{2+} , pH?) and membrane lipid content (cholesterol, acidic phospholipids).

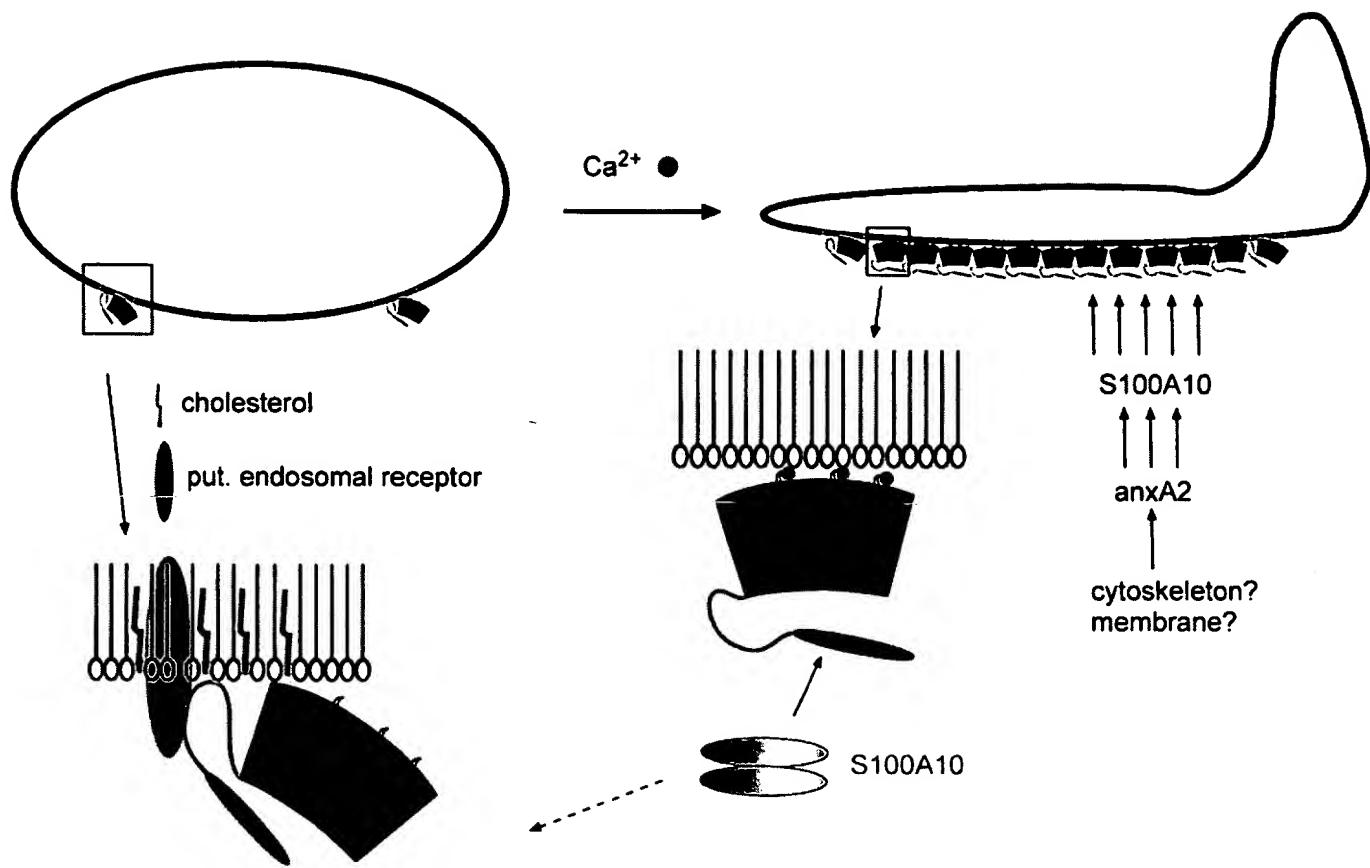


FIG. 6. Ca^{2+} -independent and Ca^{2+} -dependent interactions of annexin A2 with endosomal membranes. In the absence of Ca^{2+} , annexin A2 (blue) binds to endosomes in a manner requiring membrane cholesterol and a unique sequence in the NH_2 -terminal domain of the protein. The amount of endosome-associated annexin A2 increases significantly in the presence of Ca^{2+} , with the membrane binding now being driven by the canonical and Ca^{2+} -dependent membrane interaction. A high-density packing of annexin A2 molecules could be envisaged, which is in line with cryoelectron microscopy studies of annexin A2 bound to artificial bilayers (167). Such annexin A2 assemblies could stabilize certain membrane domains/organizations, and after S100A10-dependent formation of the annexin A2-S100A10 heterotetramer, these domains could be linked to a second membrane or cytoskeleton surface.

B. Annexins and Ion Channels

In section II B we discussed the Ca^{2+} -dependent and Ca^{2+} -independent interactions between annexins and phospholipid membranes. In the former case, such interactions depend largely on the conserved annexin core and the Ca^{2+} -binding sites therein, and in both cases a degree of reversibility is a characteristic of membrane binding for most members of the annexin family. A major challenge in the annexin field has been to understand what annexins do once membrane bound. For some annexins there is increasingly persuasive evidence for roles in vesicle trafficking and/or membrane organization, but for most annexins, the functional significance of reversible membrane binding remains elusive. However, one intriguing possibility to have emerged in the last decade is that membrane-associated annexins might function either as ion channels and/or ion channel regulators. If correct, such activities would point to annexins as both effectors

and regulators of ion fluxes and place these proteins as key mediators in the control of cellular Ca^{2+} homeostasis.

1. Regulation of ion channel activity by annexins

The first study to implicate any annexin in the regulation of ion flow demonstrated that purified annexin A6 increased the mean open time and opening frequency of the sarcoplasmic reticulum ryanodine-sensitive Ca^{2+} channel (67). This modulatory activity appeared to be specific because there was no effect of annexin A6 on sarcoplasmic K^+ or Cl^- currents. These findings suggested that annexin A6 might have a role in the regulation of intracellular Ca^{2+} signaling in cardiomyocytes and skeletal muscle cells, cell types in which annexin A6 is known to be expressed at high levels. However, annexin A6 only exhibited this property when added to the luminal side of the membrane, and annexin A6 is generally considered to be a cytosolic protein. The problem of under-

standing the effect of annexin A6 in this system was further compounded by the subsequent observation that purified annexin A6 exhibits Ca^{2+} channel activity in artificial phospholipid membranes (19). This raises the possibility that changes in the Ca^{2+} permeability observed in sarcoplasmic reticulum membranes attributed to the ryanodine receptor may have been partly or wholly due to annexin A6 itself. Another study, this time demonstrating a cytosolic effect of annexin A6, showed that the introduction of a neutralizing antibody to annexin A6 led to an increase in K^+ currents in dorsal root ganglia (DRG) and spinal cord neurons, and to an increase in Ca^{2+} currents in DRG (211). A model in which displacement of annexin A6 from intracellular membranes leads to amplification of the Ca^{2+} signal is consistent with work showing that elevation of cytosolic Ca^{2+} induced by hydroperoxide is associated with translocation of annexin A6 from the cytoplasmic face of the plasma membrane (130). Another study, again supporting a role for annexin A6 in the regulation of Ca^{2+} influx, showed that ectopic expression of annexin A6 in epithelial A431 cells leads to attenuation of Ca^{2+} influx stimulated by EGF (84). A431 cells normally lack annexin A6 and exhibit a sustained elevation of intracellular Ca^{2+} after exposure to EGF. This work showed that whereas Ca^{2+} release from intracellular stores was unaffected by annexin A6, membrane hyperpolarization and consequent Ca^{2+} influx was inhibited. Interestingly, this inhibitory activity was only observed for the larger of the two splice forms of annexin A6, an effect that also directly correlated with splice variant-specific growth suppression in these cells.

Annexins A2 and A4 have also been demonstrated to modulate the activity of ion channels, although in both cases the effects were observed on Cl^- rather than Ca^{2+} channels. Annexin A4 is located at the apical cell plasma membrane in polarized colonic T84 cells, and in electrophysiological experiments it was found that introduction of purified recombinant annexin A4 into T84 cells through the patch electrode led to inhibition of the Ca^{2+} -induced Cl^- current (146). Consistent with this, an antibody to annexin A4 and also an antisense oligonucleotide to annexin A4 mRNA, both reduced the threshold for Cl^- current activation. Activation of the Ca^{2+} -dependent Cl^- conductance occurs in response to mobilization of Ca^{2+} from intracellular stores or, in patched cell experiments, by introduction of calmodulin-dependent protein kinase II (CaMKII). Inhibition of CaMKII using a specific peptide blocked the activation of the Ca^{2+} -dependent Cl^- channel observed with the antibody to annexin A4 (37). Annexin A4 is not believed to activate or inhibit CaMKII, nor to act as a substrate for the kinase, suggesting that it may inhibit the Cl^- channel through a direct protein-protein interaction. Regulation of the Cl^- channel by annexin A4 might also involve inositol 3,4,5,6-tetrakisphosphate [$\text{Ins}(3,4,5,6)\text{P}_4$]. In electrophysiological experiments in

T84 cells, the potency of $\text{Ins}(3,4,5,6)\text{P}_4$ as an inhibitor of the Ca^{2+} -dependent Cl^- channel was doubled in the presence of annexin A4, when annexin A4 was added at a concentration at which it has no inhibitory effect when used alone (339). These observations suggest a function for annexin A4 as part of a complex signaling pathway involving both protein kinases and intracellular Ca^{2+} . The involvement of annexin A2 in Cl^- channel regulation is less well characterized but no less intriguing. A single study reported that introduction of a synthetic peptide corresponding to the NH_2 -terminal domain of annexin A2 into endothelial cells via a patch electrode led to rundown of the osmotic-regulated Cl^- channel (213). In a control experiment, a similar peptide with a single amino acid substitution that eliminated S100A10 binding was shown to be without effect. These results imply that correct assembly of the annexin A2/S100A10 heterotetramer is required for the integrity of this current. The reason for this is unclear, but in many cells the annexin A2/S100A10 complex is stably associated with the submembraneous actin cytoskeleton, which in turn may have a functional interface with volume-sensitive Cl^- channels (VSCC). Indeed, specialized membrane-cytoskeleton compartments rich in cholesterol, caveolin, and ERM (ezrin, radixin, moesin) proteins are implicated in VSCC function and also contain annexin A2 (316). Furthermore, chicken DT40 cells containing a targeted disruption of the annexin A2 gene exhibit a more hyperpolarized resting membrane potential, consistent with dysregulation of chloride homeostasis (163). Collectively, these studies suggest that whereas annexin A4 may be involved in a direct molecular interplay with the Ca^{2+} -dependent Cl^- channel, the effect of annexin A2 on VSCCs might be an indirect consequence of a possible role in the regulation of membrane cytoskeleton interactions.

2. Annexin-dependent ion fluxes

The problem of defining specific functions for individual annexins is a recurring theme in annexin research. When annexin A1 (lipocortin) was first described as an inhibitor of phospholipase A₂ (PLA₂), this was widely accepted as an explanation for the apparent anti-inflammatory activity of annexin A1. However, as the family expanded, it rapidly became clear that all annexins can inhibit PLA₂ by Ca^{2+} -dependent sequestration of the phospholipid substrate (59). Although the inhibition of PLA₂ is therefore now believed to be nonspecific and probably nonphysiological, the anti-inflammatory activity of annexin A1 is nevertheless specific to this protein, and recently a more convincing mechanism has emerged to explain its activity (see sect. IV C1). A similar history surrounds the idea that annexins might function as ion channels. The first annexin to be shown to have Ca^{2+} channel activity was annexin A7 (233, 252), but this was soon

extended to annexin A5 (253), and in conjunction with X-ray crystallographic studies, most annexins have now been demonstrated to have Ca^{2+} channel activity (19, 29, 134, 232). Just as PLA_2 inhibition by annexins was shown to be the consequence of a shared biochemical property, so it appears that the Ca^{2+} channel activity of annexins may be due to a common structural feature, namely, the central hydrophilic pore in the annexin core (see sect. II A1). However, in contrast to the anti-inflammatory activity of annexin A1, no annexin has ever been shown to exhibit Ca^{2+} channel activity in a living cell. Moreover, the general view of annexins as peripheral membrane binding proteins presents a conceptual obstacle to the idea that these proteins could function as ion channels. Despite this, the selectivity of the annexin ion channel for Ca^{2+} , together with pharmacological and electrophysiological properties that correspond to those of as yet uncharacterized Ca^{2+} channels in nonexcitable cells, ensures continuing interest in this subject.

To understand how annexins might function as Ca^{2+} channels, it is necessary to examine the way in which annexins interact with phospholipid membranes. In experiments using artificial lipid bilayers, the interaction between annexins and membranes depends on four key variables; these are the phospholipid composition of the bilayer, the concentration of the annexin, the concentration of Ca^{2+} , and the transmembrane voltage (125). For ion channel activity to occur, the annexin must first bind to the membrane, and then some poorly understood gating process must follow that leads to Ca^{2+} flux. Because the first step depends on the availability of free Ca^{2+} , the observation that as Ca^{2+} concentration increases the Ca^{2+} channel activity decreases may appear counterintuitive. However, a Ca^{2+} binding site in the proposed channel makes the current-voltage curve for annexin A5 nonlinear (253), and it is established that annexin A5 becomes more tightly membrane bound at elevated Ca^{2+} levels and that this has the effect of stabilizing the membrane (104, 210). For Ca^{2+} channel activity to occur, membrane destabilization is required. In addition, several annexins display anticooperativity of binding, such that as the protein concentration increases, so membrane binding decreases (125). Annexins also bind to membranes with higher affinity at increasingly negative resting membrane potentials, although in the absence of Ca^{2+} the membrane potential negativity required for annexin binding extends well out of the accepted physiological range. However, the requirement for a negative potential difference is reduced as the free Ca^{2+} concentration increases. In applying a reductionist approach to the analysis of annexin ion channel activity and membrane binding, one should keep in mind that some of these variables may have little influence *in vivo*, whereas other factors (such as other divalent cations, posttranslational modifications, and co-factors) could well come into play. Also, when one con-

siders the issue of annexin-specific channel activity, it is important to note that with regard to these four variables annexins behave quite differently. For example, whereas high free Ca^{2+} concentration leads to tight binding of annexin A5 to membranes, this is not the case for either annexins A6 or A7. Thus, under conditions of high free Ca^{2+} , annexins A6 and A7 display more Ca^{2+} channel activity than annexin A5 (125).

Recent studies have added a fifth key variable, namely, pH, to the parameters that govern the interactions between annexins and phospholipid membranes (see sect. II B). Several investigators have reported that mildly acidic pH favors the Ca^{2+} -independent binding of annexins to phospholipid membranes (158, 168), and spin labeling experiments using derivatized recombinant *Hydra* annexin B12 showed that protonation led to gross changes in the structure of the protein, proposed to correspond to the assembly of transmembrane α -helices which form a new membrane-spanning molecule with a central hydrophilic pore (168) (Fig. 4). Subsequent experiments showed that at pH 5.0 and below, annexins A5 and B12 both label with a photoactivatable agent that partitions into the hydrophobic domain of a lipid bilayer and that both proteins form ion channels at low pH but not at neutral pH (138). Further studies supporting the idea that annexin B12 forms a transmembrane structure showed that when added to the opposite side of the bilayer to the purified annexin, pronase caused an increase to preannexin values in the conductance of the probe nonactin (289). The conclusion of these carefully controlled studies is that the pronase effect can only be explained if annexin B12 forms a structure that spans the lipid bilayer. The key points to emerge from these studies is that the pH-dependent insertion of annexins into lipid bilayers is reversible and that it is inhibited by free Ca^{2+} . The reversibility is significant because it suggests that a dynamic equilibrium may exist between the soluble cytosolic annexin, the peripherally membrane-bound form, and the membrane-inserted form. At neutral pH it can be argued that the equilibrium is heavily biased away from the membrane-inserted form, which is consistent with reports that channel activity for annexins, despite being widely reported at neutral pH, is actually exceedingly rare (125, 138). Despite the topographical appeal of this model, there is resistance to the idea that the tightly folded α -helical annexin core could unfold and insert into membranes. Nevertheless, molecular rearrangements of this sort are not unprecedented because members of the Bcl family of pro- and antiapoptotic proteins undergo similar pH-dependent insertion into acidic phospholipid bilayers (198, 268, 271). A second problem concerns the requirement for a pH < 6.0 to induce annexin membrane insertion, given that in healthy living cells the intracellular pH probably never falls below 6.5.

In considering these problems there is a need to

interpret the findings of *in vitro* studies in terms of how annexins might function as ion channels in living cells. Thus, if annexin A5 does function as a Ca^{2+} channel, the expectation might be that such an activity would be favored under conditions of intracellular acidification and membrane hyperpolarization. In B lymphocytes, exposure to physiological concentrations of peroxide leads to a Ca^{2+} influx accompanied by membrane hyperpolarization and intracellular acidification from pH 7.3 to pH 7.0. Other Ca^{2+} mobilizing agonists such as thapsigargin or antibody to the B cell receptor do not change membrane polarity or cytosolic pH. Targeted disruption of the annexin A5 gene in B cells leads to loss of the Ca^{2+} influx component of the peroxide response, but Ca^{2+} responses to other agonists are unaffected in such cells (163). Significantly, this study also showed that in experiments using a photoactivatable agent to label annexin A5 in synthetic lipid bilayers, exposure of the protein to peroxide led to membrane insertion independently of any requirement for acidification. These results support a role for annexin A5 either as a Ca^{2+} channel or as an essential signaling intermediate in a Ca^{2+} influx pathway. Further cellular evidence for a Ca^{2+} channel role for annexin A5 comes from studies on mineralizing chondrocytes (157). The deposition of new bone by chondrocytes is mediated by matrix vesicles, PS-rich structures with a proteinaceous core that binds Ca^{2+} upon entry, to form calcium phosphate crystals. The Ca^{2+} influx channels in matrix vesicles share many properties with annexin A5 Ca^{2+} channels *in vitro*. Thus both are blocked by Zn^{2+} , activated by ATP, and inhibited by GTP, and maximal Ca^{2+} influx is observed when annexin A5 is associated Ca^{2+} -independently with the vesicles (4). These studies, together with those in B cells, provide evidence that annexin A5 may indeed function as a Ca^{2+} channel under certain conditions. However, definitive proof may be difficult to obtain, and even if this is eventually established, the question of whether or not annexin A5 functions as a peripherally bound or integral membrane protein may take longer to answer.

C. Extracellular Annexin Activities

Although annexins *per se* are intracellular proteins, i.e., they localize to the cytoplasm and/or intracellular organelles and lack signal sequences guiding them to the canonical secretory pathway, a number of properties have been attributed to extracellular annexins. Although it is still a mystery whether and to what extent regulated secretion of annexins can occur, the recent description of cell or extracellular matrix receptors for different annexins supports the physiological (or pathophysiological) meaning of such extracellular properties. The more recent findings along these lines will be summarized below. For a detailed and more complete account of extracellular

annexin activities described in the past, the reader is referred to the review by Raynal and Pollard (244).

1. Annexin A1 and the control of inflammatory responses

Annexin A1 (lipocortin I) has long been suggested to function as a cellular mediator of anti-inflammatory glucocorticoids since its expression and secretion in several cell types is induced by glucocorticoids (see, for example, Refs. 46, 228, 308) and since exogenously administered protein exhibits anti-inflammatory activities in several animal models of inflammation (for reviews, see Refs. 85, 86, 260). Although this effect has initially been attributed to the capability of annexin A1 to inhibit PLA_2 and thus the production of eicosanoids, it has become clear more recently that the anti-inflammatory activity of the protein is most likely due to interference with granulocyte recruitment, migration, and/or activation at sites of inflammation (for reviews, see Refs. 105, 221, 222).

Inflammatory processes are characterized by a localized emigration of neutrophils and other leukocytes from the blood into the inflamed tissue. This process crucially depends on strictly regulated interactions between the leukocytes and the endothelial lining of the vessels ranging from leukocyte rolling on the endothelium to firm adhesion of the blood cells and finally their transendothelial migration (for reviews, see Refs. 176, 298). The transition from rolling to firm adhesion of neutrophils requires their activation by soluble or surface-bound mediators like chemokines and other chemoattractants that also provide a gradient along which neutrophils navigate toward the site of inflammation. Glucocorticoids delay this extravasation of leukocytes, and several lines of evidence have implicated annexin A1 as a central mediator of this glucocorticoid effect. In neutrophils adhering to activated endothelium, annexin A1 is mobilized and externalized through an as yet unknown mechanism resulting in a downregulation of neutrophil transmigration (224). Most likely this is due to an inhibitory effect on transmigration of the released annexin A1, which has been described *in vitro* and *in vivo* with exogenously applied protein as well as peptides derived from the unique NH_2 -terminal domain of annexin A1 (for reviews, see Refs. 105, 221, 222). Such NH_2 -terminal peptides typically covering residues 1–25 of the annexin A1 sequence in their NH_2 -terminally acetylated form (Ac1–25), as well as the entire molecule, are also capable of restricting leukocyte migration and thus tissue damage in animal models of splanchnic artery occlusion/reperfusion and myocardial ischemia/reperfusion injury (56, 58). Thus *in vitro* systems as well as a number of animal models have proven the antimigratory effect of annexin A1 (and the unique NH_2 -terminal peptides) on leukocyte extravasation, and the protective influence of the protein/peptides in several pathophysiological situa-

tions has sparked interest in their pharmacological potential.

Given these well-established effects of annexin A1 on neutrophil extravasation, two questions arise. First, what is the mechanistic basis of the effect, and second, can endogenous annexin A1 function as a modulator of leukocyte extravasation, e.g., by downregulating inflammatory responses to prevent chronic inflammation? To explain the antimigratory effects, a direct action of extracellular annexin A1 on leukocytes had long been postulated and indeed been proven in a number of studies. Among other things, the NH₂-terminal annexin A1 peptide Ac1-25 was shown to affect directly a number of neutrophil functions, and preincubation of neutrophils with the annexin A1 peptide resulted in decreased migration of these cells through untreated endothelial monolayers (226, 331). Proteinaceous binding sites for annexin A1 have been shown to exist on human monocytes and neutrophils as well as on monocytic U937 cells, with bound annexin A1 in the latter case being colocalized and coimmunoprecipitated with $\alpha_4\beta_1$ -integrins (75, 291). Compelling evidence has been obtained recently for a specific binding of annexin A1 (and its NH₂-terminal peptides) to the formyl peptide receptor (FPR) on neutrophils. FPR is a heptahelical, G protein-coupled receptor recognized by bacterial peptides of the prototype formyl-Met-Leu-Phe (fMLP) which are thought to provide the chemoattractant gradient for guiding neutrophils toward the site of bacterial infection. When employed in an *in vitro* transmigration model, antagonistic fMLP peptides were shown to reverse the inhibitory effect of annexin A1 (and its NH₂-terminal peptide) on neutrophil transmigration, and ectopically expressed FPR was specifically triggered by the annexin A1 peptides (331). As the annexin A1 peptides can also desensitize the FPR on neutrophils toward fMLP challenge, it appears that their inhibitory action on neutrophil extravasation is based on their binding to and activation/desensitization of the FPR. Such a role of the FPR in mediating the anti-inflammatory effect of annexin A1 has been corroborated by *in vivo* experiments employing FPR knockout mice (225). FPR activation by annexin A1 peptides can occur also in nonmyeloid cells leading, e.g., in human lung A549 cells, to the induction of acute phase protein expression, thus arguing for a more widespread role of the annexin A1-FPR interaction (U. Rescher, A. Danielczyk, A. Markoff, and V. Gerke, unpublished observations).

Although this provides a mechanistic explanation for the pharmacological action of exogenously applied annexin A1 (or NH₂-terminal annexin A1 peptides), it is not clear whether such scenarios could also hold true for endogenously released annexin A1. Regulated externalization of annexin A1 has been described for endothelium-adherent leukocytes (224, 291), but it is not clear whether the amounts released are sufficient to trigger the

FPR. Moreover, with the mechanism of secretion unknown, it remains possible that the extracellular annexin A1 stems at least in part from lysed cells. Thus we are in need of identifying in molecular terms a system promoting regulated annexin A1 release. The protein does not follow the classical secretory pathway because its release is not inhibited by brefeldin A, monensin, or nocodazole (228). However, in neutrophils, some annexin A1 colocalizes with gelatinase in gelatinase storage granules, and this appears to be released to the cell surface upon adhesion of the neutrophils to endothelial monolayers (223). Nonetheless, it is not clear how the protein reaches the lumen of the granules and whether this relates to the Ca²⁺-dependent secretion of annexin A11 in activated human neutrophils, which was described recently in a proteomic approach (25). Whatever model is favored, for active secretion to occur the transport of annexin A1 across the bilayer (that of the plasma membrane or of internal membranes of secretory organelles) is required, and it is not yet clear how that can happen. In this context, the pH-induced conformational change allowing annexin A1 to insert into the bilayer (see above) might be hypothesized, but it remains to be shown whether such insertion (possibly followed by a release at the other side of the bilayer) can occur under physiological conditions met in cells.

2. Extracellular activities of other annexins

This section focuses on recent developments relating to extracellular activities of annexins A2 and A5. Extracellular annexin A2 has been described as a surface-bound receptor for a number of different molecules, indicating that it might act as a more general surface anchor and not as a specific receptor of a given ligand. Best studied is probably the interaction of elements of the plasmin/plasminogen activator system with annexin A2 present on the surface of endothelial cells. Annexin A2 binds both plasminogen and the tissue plasminogen activator (tPA), with the former interaction being inhibited by the atherogenic lipoprotein A and the latter being blocked by homocysteine (for review, see Ref. 114). The tPA binding site on annexin A2 has been mapped to the NH₂-terminal domain and encompasses residues 7–12. This sequence contains an accessible cysteine residue at position 8 (142), and it is this cysteine that is derivatized by homocysteine leading to the reduction in tPA binding (112). Thus annexin A2 present on the surface of endothelial cells could play a role in fibrinolytic surveillance by anchoring key components of the fibrinolytic cascade. Increased homocysteine levels would interfere with this annexin A2 action, thus providing a possible explanation for a link between increased homocysteine levels and the risk of atherosclerosis (113). The antithrombotic action of annexin A2 could also be directly associated with the

hemorrhagic disorder in patients suffering from acute promyelocytic leukemia, as will be discussed below. However, we have to take into account that annexin A2 is probably not the only cell surface receptor for tPA on the endothelium (90). Moreover, in addition to binding to tPA and stimulating the conversion of plasminogen to plasmin, annexin A2 (in complex with its S100A10 ligand) can also inhibit the plasmin-mediated lysis of fibrin polymers and can inhibit plasmin activity by stimulating its auto-proteolytic digestion (41, 42, 82). Thus the relation between extracellular annexin A2 and the regulation of thrombogenesis is likely to be complex.

Annexin A2 present on the surface of endothelial cells has also been described to represent the binding site for β_2 -glycoprotein I, a phospholipid-binding protein from plasma known as an autoantigen in the antiphospholipid antibody syndrome. This interaction indicates that the association of β_2 -glycoprotein I with endothelial cells is not mediated directly by phospholipids but depends on annexin A2 (186). Other ligands for cell-surface bound annexin A2 are procathepsin B (on human breast carcinoma and glioma cells) (187) and a vitamin D analog that appears to use annexin A2 as a receptor on rat osteoblast-like cells with the interaction being inhibited by Ca^{2+} (12, 13). It is not clear whether and how this relates to the finding that gene expression of annexin A2 itself is up-regulated by 1,25-dihydroxyvitamin D₃ and that extracellular annexin A2 stimulates the proliferation of osteoclast precursors by activating T cells to secrete granulocyte-macrophage colony stimulating factor (194). However, as already discussed above for annexin A1, it remains to be shown whether, how, and to what extent annexin A2 is actively exported from, for example, endothelial cells and whether such export can be regulated under physiological conditions. Moreover, for annexin A2 acting as a cell surface receptor, it requires anchoring on or in the cell membrane, the mechanism for which has so far not been elucidated.

Several functions have also been proposed for extracellular annexin A5. It was originally described as an anticoagulant protein, and this activity most likely depends on its Ca^{2+} -regulated binding to anionic phospholipids, possibly those exposed on the surface of activated platelets or endothelial cells. This binding could interfere with the accessibility of such sites for coagulation factors, thereby preventing their local accumulation/activation (for review, see Ref. 244). More recently, antibody-mediated inhibition of an anticoagulant property of annexin A5 has been proposed to occur in recurrent pregnancy losses observed in patients with antiphospholipid syndrome (for review, see Ref. 240). As discussed in detail in section v, annexin A5 binds to the apical surface of placental syncytiotrophoblasts and by shielding these coagulation-promoting surfaces could be important for the maintenance of blood flow through the placenta. Anti-annexin A5 anti-

bodies that are found in patients with antiphospholipid syndrome (and also in the sera of patients suffering from systemic lupus erythematosus, Ref. 266) decrease the ability of annexin A5 to form a shield on the trophoblast surface and could thus cause placental thrombosis (240, 241, 243, 333). In vitro annexin A5 was also shown to interact with the NH₂-terminal (extracellular) domain of polycystin 1, the major protein affected in autosomal dominant polycystic kidney disease, although the in vivo relevance of this interaction remains to be established (A. Markoff, N. Bogdanova, U. Rescher, F. Qian, B. Dworznickzak, G. Germino, Y. Horst, and V. Gerke, unpublished observations). Moreover, the protein is capable of binding to components of the extracellular matrix, in particular types II and X collagens. Such binding could relate to the finding that annexin A5 (as a Ca^{2+} channel, see above) affects the Ca^{2+} uptake in chondrocyte-derived matrix vesicles in a manner depending on the binding to collagens types II and X (for review, see Ref. 329). The annexin A5 knock-out models underway should answer the question whether annexin A5 has a crucial role to play in such processes.

D. Annexin Transgenesis and Targeted Gene Disruption

Modulation of gene expression by either transgenic expression or targeted gene disruption has been used in many species to gain insight into protein function. Given the historical difficulty of assigning functions to annexins, it is perhaps surprising that such studies appeared relatively recently in the annexin literature. This might have been partly due to the expectation that annexins would be discovered in yeast, in which genetic manipulation is facile, or could reflect an unwillingness by funding agencies to support what are seen to be risky projects. Despite the absence of annexins in yeast, the presence of annexins in roundworms and insects leads one to hope that eventually mutants will be established in these organisms that might give clues to function. Indeed, the first genetic experiment involving annexins reported that disruption of annexin C1 in *Dictyostelium* did not lead to any adverse effects when cells were cultured under normal conditions (71). However, the cells were significantly disadvantaged when cultured in low external Ca^{2+} , exhibiting defects in growth, motility, and chemotaxis, observations that support a role for annexin C1 either as a Ca^{2+} mediator or as a regulator of Ca^{2+} homeostasis (72). The only transgenic studies reported to date describe the effects of overexpression of annexin A6 targeted to cardiomyocytes. These animals display left ventricular dilation and cardiomyopathy and die of heart failure at a relatively young age. Studies on isolated cardiomyocytes from young animals revealed that overexpression of annexin A6 was associ-

ated with a lower resting level of cytosolic Ca^{2+} and smaller Ca^{2+} spikes associated with attenuation of contractility when the cardiomyocytes were electronically paced (109). These results correlate inversely with studies on annexin A6 expression in failing human hearts (described in sect. vA2). Perhaps unexpectedly in view of these results, annexin A6 null mutant mice are healthy and fertile and fail to exhibit any cardiovascular defects with regard to heart rate, blood pressure, and circulatory collapse in response to endotoxic shock (121). However, in certain respects, the mouse is not an ideal model for human cardiovascular function, partly because the mouse heart beats almost maximally so that any "enhancement" in cardiomyocyte contractility would be difficult to detect. Indeed, in isolated cardiomyocytes from annexin A6 knock-out mice in which contraction rate can be regulated, significantly increased mechanical properties linked to altered Ca^{2+} handling are observed, when compared with cells from wild-type littermates (G. Song, S. E. Moss, and M. Duchen, unpublished observations).

The lack of an overt phenotype in annexin A6 knock-out mice contrasts with studies on annexin A7. Targeted disruption of annexin A7 in mice led to embryonic lethality at *day 10* due to cerebral hemorrhage. Mice heterozygous for the mutation are viable and fertile but have defects in insulin secretion, although the insulin content of islet cells is considerably higher than in wild-type mice (299). Investigation of this phenotype revealed that expression of the inositol 1,4,5-trisphosphate (InsP_3) receptor was also reduced, leading to the failure of InsP_3 to release intracellular calcium. Although this observation explains the phenotype and establishes a potential link between annexin A7 and Ca^{2+} signaling, it is not clear why a partial reduction in annexin A7 expression levels should be accompanied by a parallel loss of InsP_3 receptor expression. In a separate annexin A7 gene knock-out project, the null mutant mice were found to be healthy and viable and no different from control mice with regard to glucose-stimulated insulin secretion, although cardiomyocytes isolated from these mice showed alterations in their frequency-induced shortening (122). However, as observed with annexin A6 knock-out mice, cardiomyocytes lacking annexin A7 manifested disturbances in power-contraction frequency. Although there are other examples of gene disruption studies in which different groups reported distinct phenotypes, and in the case of annexin A7 there were differences in design of targeting constructs and disruption sites, reconciliation of the embryonic lethality and minimal phenotype reported by these two groups represents a considerable challenge. In other ongoing studies, mice containing a targeted disruption of the annexin A1 gene are reported to be viable and healthy (R. Flower, personal communication), and matings between mice heterozygous for a disrupting mutation of the annexin A5 gene yield viable pups (K. von der Mark and E.

Pöschl, personal communication). Although systematic analysis of annexin null mutant mice is therefore still at an early stage, these preliminary observations exemplify not only the potential value of gene knockout in exploring annexin function, but also the need to keep an open mind with regard to the interpretation of phenotype.

V. ANNEXINS AND HUMAN DISEASE

A. Disorders of the Heart and Circulation

1. Annexins and cardiovascular biology

As yet, no human diseases have been described in which a mutation in an annexin gene is a primary cause. However, there is evidence that through changes in expression, properties, or localization, annexins may contribute to the pathophysiology of disease phenotypes. The most striking examples of these secondary effects have been termed "annexinopathies" (239) and are characterized by dysregulation of what may be the normal anti-thrombotic properties of extracellular annexins. As discussed earlier, annexin A2 on the surface of vascular endothelial cells can act as a receptor for tPA, so its presence would favor a thrombolytic environment and might therefore make a positive contribution to the overall health of the vasculature. Conversely, changes in endothelial cell behavior leading to reduced cell surface expression of annexin A2 or metabolic changes that chemically modify annexin A2 could be hypothesized to lead to predisposition to cardiovascular disease. Of numerous risk factors implicated in atherothrombotic vascular disease, elevated plasma homocysteine is of particular relevance. Homocysteine is a metabolic derivative of dietary methionine and was shown to incorporate into the NH_2 terminus of annexin A2 replacing Cys-8, a key residue in mediating the tPA processive activity of annexin A2 (113). In this study, substitution of Cys-8 with homocysteine led to a ~65% loss in tPA binding capacity, consistent with the development of reduced thromboresistance in homocysteinemic individuals. A second risk factor with a well-established link to cardiovascular disease is oxidative stress. In a recent study, annexin A2 was identified as a major cellular target for glutathiolation in response to oxidative stress induced by hydrogen peroxide or $\text{TNF-}\alpha$. Interestingly, the reactive cysteine identified as the target for glutathione in this study was again Cys-8 in the annexin A2 NH_2 terminus (304). A third risk factor shown in epidemiological studies to be linked to cardiovascular disease is alcohol consumption, although the effect of moderate intake is protective rather than deleterious. One recent study reported that vascular endothelial cells cultured in the presence of low concentrations of ethanol exhibited a doubling of cell surface fibrinolytic activity

that correlated with a sustained increase in annexin A2 mRNA and protein (306). Although these studies do not prove that annexin A2 is directly involved in the development of or susceptibility to cardiovascular disease, they support the idea that metabolic changes known to influence risk could be mediated at least in part by chemical modifications in and transcriptional regulation of annexin A2.

2. Heart disease

Because of the importance of Ca^{2+} homeostasis in the heart, and the abundance of annexins A2, A5, and A6 in cardiomyocytes and the supporting cellular infrastructure, there is considerable interest in elucidating the roles of cardiac annexins. Immunocytochemical studies identified annexins A5 and A6 in both myocytes and nonmyocytes in a variety of species, and most of these reported a concentration of annexin A5 with the sarcolemma and Z line in cardiomyocytes (141, 183, 191, 317, 332) and a preferential localization of annexin A6 with the sarcolemma and intercalated disks (184, 191, 317). In addition, annexin A5 has been reported to be localized to the nucleus and nuclear membrane in neonatal differentiating myocytes and becomes associated with the sarcolemma only in terminally differentiated adult cells (183). In a parallel study, annexin A6 was found colocalized with uncharacterized subcellular structures in neonatal myocytes and was only associated with the sarcolemma in adult cells (184). Although the function of cardiac annexin A5 is not known, the benzothiazepine derivative K201, which blocks the Ca^{2+} channel activity of annexin A5 in vitro (150), has been shown to protect the myocardium against the cytotoxic effects of Ca^{2+} associated with ischemia/reperfusion injury (110).

Several other lines of evidence suggest that annexins have important functions in the heart. The most striking of these is the demonstration that cardiomyocyte-specific overexpression of annexin A6 in transgenic mice leads to hypertrophy and heart failure (109) (see also sect. ivD). This study prompted several investigations into annexin expression in heart disease, both in a variety of animal models and also in humans with end-stage heart failure. The picture that emerges from these studies is that the expression levels of annexins A2, A5, and A6 are largely unaffected during ventricular hypertrophy (141), but that during end-stage heart failure the levels of annexin A6 fall in cardiomyocytes whereas those of annexins A2 and A5 rise (292). Other investigators reported similar rises in annexins A2 and A5 during heart failure, but restricted to nonmuscle cells (18), and also elevation of annexin A6 in interstitial tissue (317). The significance of these changes is not clear, but given that overexpression of annexin A6 reduces the contractility of cardiomyocytes (109) and that the opposite effect is observed in annexin A6 null mutant

mice (Song et al., unpublished observations), it is possible that downregulation of annexin A6 during heart failure is a form of molecular compensation that favors improved cardiomyocyte function.

3. Annexins as anticoagulants

As discussed in section vA1, there is growing evidence that annexin A2 has an antithrombotic role at the endothelial cell surface. More direct evidence for the involvement of annexin A2 in disease pathology emerged from studies on leukemic cells from patients with acute promyelocytic leukemia (APL). Patients with APL exhibit an increased tendency to hemorrhagic diathesis and respond well to treatment with *all-trans*-retinoic acid. APL leukocytes were found to strongly overexpress annexin A2 at the cell surface and also to stimulate the generation of plasmin from tPA twice as efficiently as other leukemic cells (195). Plasmin generation was blocked by anti-annexin A2 antibodies and could be induced in non-APL cells by ectopic expression of annexin A2. Moreover, exposure of APL cells to *all-trans*-retinoic acid led to a marked reduction in annexin A2 mRNA and protein which correlated with diminished tPA binding. This study provides the clearest evidence so far for any member of the annexin family having a direct role in the pathophysiology of a human disease.

Like annexin A2, annexin A5 has also been suggested to have an antithrombotic role that becomes compromised in disease. However, whereas annexin A2 appears to function as an intermediary in the fibrinolytic cascade, annexin A5 has been proposed to have a more direct role, by forming a molecular shield that insulates the apical surfaces of placental villi from the activities of circulating coagulant proteins. Anticoagulant activity is a feature of all Ca^{2+} binding annexins and can be explained by simple Ca^{2+} -dependent sequestration of the phospholipid matrix with which procoagulant factors interact. In this respect, annexins behave in exactly the same way as when first described as inhibitors of PLA_2 , since the enzyme also requires both Ca^{2+} and phospholipid as cofactor and substrate, respectively. Although this type of anticoagulant activity and PLA_2 inhibition have long been viewed as purely in vitro properties of annexins, there is now evidence that the anticoagulant activity of annexin A5 might be of biological importance in the recurrent pregnancy losses associated with antiphospholipid syndrome (240). A diagnostic observation in patients with antiphospholipid syndrome is the presence in the serum of antibodies against a range of proteins or phospholipids, including annexin A5, prothrombin, cardiolipin, β_2 -glycoprotein-I, and phosphatidylethanolamine (78, 314), and a number of studies suggest that displacement of the annexin A5 shield by anti-annexin A5 antibodies is causative in the generation of a thrombogenic environment and consequent fetal loss. For example, displacement of annexin A5

from the syncytiotrophoblast surface with either specific antisera or Ca^{2+} chelator (242) leads to accelerated coagulation of plasma, and in a mouse model, infusion of anti-annexin A5 antibodies led to placental infarction and pregnancy wastage (333). However, not all investigators take the same view. Several clinical studies failed to detect either anti-annexin A5 antibodies (278) or any changes in expression or localization of annexin A5 (166) in women with pregnancy loss associated with antiphospholipid syndrome. Another study found no evidence that displacement of annexin A5 using antiphospholipid antibodies increased the thrombogenicity of the cell surface (21). If annexin A5 really does have a protective function in the placenta as an anticoagulant, the mechanism of its activity is likely to be considerably more complex than proposed in current models, suggesting that the protein forms an antithrombotic shield in two-dimensional crystalline arrays on the exposed phospholipid surface.

B. Annexins and Physiological Stress

Physiological stress occurs at the cellular level in many disease states and is typically associated with the activation of certain signaling pathways, changes in cell morphology and activity, and modulation of gene expression. Many of these changes can be induced in normal cells by using osmotic, temperature, and oxidative shock, and new research suggests that members of the annexin family may be involved in the cellular response to stress. Annexin A1 was reported to have chaperone activity in *in vitro* experiments in which the purified protein was demonstrated to protect the enzymes citrate synthase and glutamate dehydrogenase from heat inactivation (155). Furthermore, heat shock, hydrogen peroxide, and sodium arsenite were all demonstrated to induce expression of annexin A1 and also translocation of the protein from the cytosol to the nucleus in A549 and HeLa cells (250). The same set of agonists also activated the annexin A1 gene promoter in experiments using a reporter gene. Other annexins have also been shown to be regulated by cytotoxic stress. For example, annexin A4 was shown to be induced in human non-small-cell lung carcinoma cells by the antimitotic drug paclitaxel and to concentrate in the nuclei of stressed cells (117). The correlation between annexin translocation to the nucleus and cellular stress extends to annexin A5, which exhibits the same behavior in primary cultures of vascular endothelial cells grown under conditions of mild hyperoxidative stress (S. M. Sacre and S. E. Moss, unpublished observations). Annexin A5 has also been identified in a screen for proteins induced by hypoxic stress in cultured human cervical epithelial cells (65). Annexin A2 was also found to be induced by hypoxia in this study and by hyperoxidative stress in a model of renal cell carcinoma (307). Thus, at

least in certain cell types, annexins A2 and A5 appear to be upregulated by changes in cellular redox state, irrespective of whether these tend toward a more reducing or a more oxidative environment. The idea that members of the annexin family might have stress-related functions is also supported by studies in plants (100, 162, 310). One of these studies reported that an annexin from *Arabidopsis thaliana* possesses catalase activity and that expression of this annexin restored the ability of a delta oxyR mutant strain of *E. coli* to grow in the presence of peroxide (100). Although such enzyme activity has never been convincingly demonstrated for any animal annexin, there is growing evidence that annexin function may be directly influenced by oxidative and perhaps other stresses. Annexin A2 is glutathiolated in HeLa cells exposed to peroxide (304), annexin A6 has been reported to contribute to the Ca^{2+} signal in macrophages exposed to peroxide by dissociation from the plasma membrane (130), and targeted disruption of the annexin A5 gene in B cells leads to loss of the Ca^{2+} influx response to peroxide (163). Mechanical stress has also been shown to influence annexin behavior. Relaxation of human foreskin fibroblasts grown on collagen matrices led to the enrichment of annexins A2 and A6 with shed membrane vesicles (173), and mild hyperosmolar shock leads to the association of GFP-tagged annexin A2 with mobile endocytic vesicles in rat basophilic leukemia cells (196). Collectively, these studies suggest that physiological stress may be important in the regulation not only of annexin gene expression but also the activities and intracellular localization of at least some annexins.

C. Annexins and Cancer

Annexins A1 and A2 were first discovered as major cellular substrates for phosphorylation on tyrosine by the EGF receptor and the transforming gene product of the Rous sarcoma virus, respectively, implicating these proteins in signaling pathways known to be subverted or involved in cancer. Nevertheless, evidence in support of causative roles for any annexin in the development of cancer or in cell transformation is still mainly circumstantial. Recent studies have reported a correlation between the level of annexin A1 expression in RAW 264.7 macrophages and the cellular responsiveness to lipopolysaccharide (LPS) of components of the mitogen-activated protein (MAP) kinase pathway (1). Cells expressing reduced levels of annexin A1 exhibited potentiation of LPS-induced MAP kinase activation, with elevated annexin A1 expression having the inverse correlation. A similar investigation reported a correlation between annexin A1 expression and mobilization of intracellular Ca^{2+} in MCF-7 breast carcinoma cells (89). In this case, overexpression of annexin A1 led to abrogation of Ca^{2+} release after activation of purinergic or bradykinin receptors, whereas

downregulation of annexin A1 using antisense had the converse effect. Another recent study provided evidence that annexin A1 overexpression in rat 2 fibroblasts leads to direct inhibition of cytosolic PLA₂, which in turn depresses the serum response element of c-fos (215). These authors also used deletion mutants to map the functional site in annexin A1 to a domain comprising the first conserved annexin repeat. Collectively, these studies all imply a growth-suppressive role for annexin A1 despite the apparent mechanistic diversity underlying each case. The difficulty comes in reconciling the reported effects of annexin A1 on MAP kinase signaling, c-fos induction, and Ca²⁺ mobilization to a single function. Does annexin A1 really regulate signal transduction pathways via interactions with multiple cellular targets, or can these observations be explained by a more general effect of annexin A1 on Ca²⁺ signaling or endocytosis? And, the conclusions of these studies are not supported by work showing that annexin A1 is strongly upregulated in a prostate cancer cell line (324), esophageal cancer (74), a stomach cancer cell line (283), mammary adenocarcinoma (220), and hepatocarcinoma (62). Interestingly, the latter study also showed that annexin A1 is upregulated during normal hepatocyte proliferation after partial hepatectomy and that the proliferative rate of both normal and malignant hepatocytes was attenuated by antisense to annexin A1. These results suggest a link between annexin A1 and cell proliferation, rather than malignant transformation per se, and they suggest that cell growth is associated with elevated rather than reduced levels of annexin A1. It must be hoped that clarification of these apparently contradictory lines of evidence will come from analysis of annexin A1 null mutant mice.

Other annexins have also been linked with cell growth and transformation, frequently in studies using similar experimental designs to those described above for annexin A1. For example, overexpression of annexin A5 in MCF-7 cells leads to inhibition of phorbol ester-mediated activation of the MAP kinase pathway (264), and heterologous expression of annexin A6 at physiological levels in human A431 carcinoma cells leads to inhibition of growth factor-mediated Ca²⁺ influx (84) and slower tumor growth in mice (309). Interestingly, the correlation between expression of annexin A6 and growth suppression extends to melanoma, where a genetic screen identified annexin A6 as a protein downregulated in the transition from a nonmetastatic to a metastatic phenotype (87). A similar result was recently obtained for annexin 2, which appears to be downregulated in prostate cancer (39), and annexin A7, which is expressed at low levels in the most metastatic malignant melanomas (153). Interestingly, a recent study examining loss of heterozygosity (LOH) at the annexin A7 locus in prostate cancer specimens identified this LOH in 35% of the primary prostate tumors. Analysis of annexin A7 expression in prostate

tumor microarrays revealed low levels of expression in metastatic and local recurrences of hormone refractory prostate cancer compared with primary tumors. Moreover, the same study showed that ectopic expression of annexin A7 in two prostate tumor cell lines reduced cell proliferation and that heterozygous annexin A7 knock-out mice (+/-) have a more cancer-prone phenotype (300). A potential role of an annexin as tumor suppressor gene is not without precedent, since Theobald et al. (309) had reported that annexin A6 has tumor suppressor activity in human A 431 cells.

The advent of global gene expression analysis using proteomics and DNA chip technology has also revealed changes in annexin gene expression in numerous cancers and other diseased or stressed cell states (36, 74, 283, 325, 337). There are several searchable web sites reporting these findings, and for one of the most informative, interested readers are referred to <http://genome-www.stanford.edu/> for the results of the NC160 Cancer Microarray Project. Some of the most striking findings here include upregulation of annexin A5 in melanomas and annexin A9 in prostate and colon cancers and downregulation of annexin A5 in leukemias and annexin A1 in prostate cancers. Correlations of this type are intriguing and suggest that changes in the levels of expression of certain annexins may influence patterns of cellular behavior, such as motility, invasiveness, and proliferative rate, without actually initiating the transformation process. As such, annexins may yet prove to have therapeutic potential in the treatment of malignant disease.

VI. CONCLUSION

Annexins comprise a multigene family of Ca²⁺-regulated membrane binding proteins that has evolved into different branches with members expressed widely throughout the animal and plant kingdoms. The conserved Ca²⁺/membrane binding unit present in all annexins (the core domain) can be viewed as a tool invented by nature to peripherally dock proteins to membranes. Such docking can occur at high density, possibly enabling the annexins to organize membranes, e.g., by assembling interacting phospholipids into certain domains, or at low density, under which circumstances annexins may increase membrane permeability. Membrane insertion shown for some annexins to occur *in vitro* at lower pH might follow such peripheral association, but conditions possibly inducing this *in vivo* and potential physiological consequences still need to be established. The second principal annexin domain located at the NH₂-terminal end is unique for a given member and specifies or fine-tunes its intracellular (in some cases also extracellular) interactions with certain target membranes or protein ligands. Such interactions may either affect annexin properties or

may be affected by annexin binding. The basis of annexin function as a whole most likely resides in their unique mode of membrane interaction, which in turn can influence a number of membrane-related events, e.g., membrane traffic and the organization of compartment membranes and the plasma membrane. Through their apparent ability to organize, perturb, or integrate into membranes with which they interact, annexins may therefore have roles as effectors, regulators, and mediators of Ca^{2+} signals. Such biological activities have now been shown for some annexins, and further knockout as well as mutant models are under development to decipher the roles of other members of the family. However, we still have a long way to go to understand the precise functions of individual annexins. Redundancy in the family coupled to the problem of dealing with scaffolding or structural functions for many annexins will demand imaginative experimental approaches and rigorous objectivity in the interpretation of the results.

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